

Studies on the relationships between bovine virus  
diarrhoea virus and border disease virus

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DECLARATION

This thesis was composed by myself. Some of the work reported was part of a larger project concerned with the pathogenesis of border disease and consequently many of the experimental findings were obtained in collaboration with colleagues at the Moredun Research Institute. Nevertheless, most of the work presented in this thesis was carried out by myself and where conjoint experiments were undertaken, a full role was played in the design of the experiments and the interpretation of the results.

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ABBREVIATIONS

AP	- Alternative pathology
BEB	- Bovine embryonic brain
BEK	- Bovine embryonic kidney
BES	- Bovine embryonic skin
BET	- Bovine embryonic testis
BD(V)	- Border disease (virus) e.g. BDV-M, Moredun strain of border disease virus
BSA	- Bovine serum albumin
BSS	- Basic salt solution
BVD(V)	- Bovine virus diarrhoea (virus) e.g. BVDV-NADL Reference Strain of bovine diarrhoea virus from National Animal Diseases Laboratory.
CF	- Complement fixation
CK	- Calf kidney
CNS	- Central nervous system
CP	- Cytopathic
CPE	- Cytopathic effect
EM	- Electron microscopy
FBS	- Foetal bovine serum
FLB	- Foetal lamb brain
FLK	- Foetal lamb kidney
FLS	- Foetal lamb skin
FLT	- Foetal lamb testis
HC(V)	- Hog cholera (virus)
HSD	- Honestly significant difference
ID(T)	- Immunodiffusion (test)

IEP	- Immunoelectrophoresis
(I)IFT	- (Indirect) Immunofluorescence test
LAH	- Lactalbumin hydrolysate
MCHC	- Mean corpuscular haemoglobin concentration
MCV	- Mean corpuscular volume
MEM	- Minumun essential medium
moi	- Multiplicity of infection
NBCS	- Newborn calf serum
NCP	- Non-cytopathic
PBS	- Phosphate buffered saline
PCV	- Packed cell volume
pi	- Post-infection
RHS	- Recovered 'hairy shaker'
RSB	- Reticulocyte standard buffer
SA	- Soluble antigen
SN	- Serum neutralisation
TCID <sub>50</sub>	- Tissue culture infective dose <sub>50</sub>
VTM	- Virus transport medium
WB	- Washing buffer

# UNIVERSITY OF EDINBURGH

## ABSTRACT OF THESIS (Regulation 7.9)

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A study was made of some aspects of the pathogenic and antigenic relationships between 2 reference strains of cytopathic (CP) pestivirus, namely bovine virus diarrhoea (NADL strain) (BVDV-NADL) and border disease virus (Moredun strain) (BDV-M).

Initial in vitro studies showed that only cultured cells of bovine and ovine origin allowed virus growth with the production of cytopathic effect. BVDV-NADL gave comparable levels of virus in various foetal tissues from both species, whereas ovine foetal tissues were superior for the growth and assay of BDV-M. Ovine foetuses had the added advantage that only 2 of 105 (1.9%) abattoir foetuses were contaminated with noncytopathic (NCP) pestiviruses as against 15 of 113 (13.3%) bovine foetuses.

A severe acute disease resembling bovine mucosal disease was produced in 7 of 8 recovered hairy shaker (RHS) sheep which were persistently infected with BDV and subsequently superinfected with high tissue culture passage BDV-M derived from the infectious brain pool used to infect their dams at 54 days' gestation. No disease occurred in RHS from the same origin superinfected with BVDV-NADL or left unchallenged.

Superinfection with low passage BDV-M of RHS from field outbreaks of BD produced serious acute or chronic disease in 4 of 10 cases but in none of 5 normal controls receiving the same inoculum. All surviving RHS lambs seroconverted to BDV-M but NCP pestiviraemia was sustained and the animals remained infectious to other lambs.

Low tissue culture passage BDV-M was used to infect 15 pregnant ewes at 54 days' gestation. Five produced 9 viable lambs, all of which had clinical BD and an NCP pestiviraemia at birth. At 9 weeks of age a set of twins was killed after exhibiting a mucosal disease-like syndrome, and CP BD virus was isolated from many organs. The twins were the only live progeny of one of the 2 tups used as sires. No such disease occurred in the other RHS in the same pen.

The pathogenicity for the placenta and foetuses of ewes infected with plaque purified stocks of the 2 reference strains or of 3 field isolates of CP BVDV was very mild. Failure to recover virus easily was attributed to poor replication of these stocks in vivo.

Antisera to the plaque purified viruses were produced in gnotobiotic lambs. By cross-immunofluorescence the 5 viruses were indistinguishable whereas cross-neutralisation segregated BDV-M as a distinct serotype from the 4 BVD viruses. Western blotting demonstrated 2 virus-induced proteins (78K and 35K) in cells infected with BVDV but only the 78K protein in cells infected with BDV-M. Antiserum to this virus did not detect the 35K protein.

CHAPTER 1REVIEW OF LITERATUREINTRODUCTION

Bovine virus diarrhoea (BVD) virus was first isolated in North America from an outbreak of enteric disease in cattle (Baker et al., 1954). Border disease (BD), a congenital disease of sheep first described in the border region of England and Wales, was later shown to be caused by a virus serologically related to BVD virus (Acland et al., 1972; Hamilton and Timoney, 1972).

An earlier important finding was that viruses from cases of BVD (synonym: mucosal disease) were serologically related to viruses isolated from cases of hog cholera (synonym: swine fever), a fatal systemic disease of pigs (Darbyshire, 1960; 1962). These three viruses have been grouped in the genus Pestivirus in the family Togaviridae (Porterfield et al., 1978). Pestiviruses have a world-wide distribution and are a significant cause of disease among susceptible domestic livestock. In Britain, HC has been eradicated by a slaughter policy, but no means of control of cattle and sheep diseases caused by pestiviruses has yet been introduced. Effective control measures rely on a knowledge of the pathogenesis of infections with these viruses and possible variations in the antigenicity of BVD and BD virus isolates. Studies in this thesis are on the pathogenic and antigenic relationships between the two viruses. In the following review of the extensive literature on BVD and BD viruses emphasis has been placed on work relevant to these areas. Other aspects of

infections with the two viruses have been included in other works (Pritchard,1963; Saurat et al.,1972; Horzinek,1981; Barlow and Patterson,1982). This review is of reports published before 1983; relevant papers published since then will be included in the discussion of chapters to which they relate.

#### FIRST DESCRIPTIONS AND NOMENCLATURE.

Virus diarrhoea, 'X' disease and mucosal disease were the names given to apparently new transmissible enteric diseases of cattle first described in different regions of North America (Olafson et al.,1946; Childs,1946; Ramsey and Chivers,1953). The terms virus diarrhoea or BVD and mucosal disease(MD) were sustained in early reports from 18 other countries around the world (Mills et al.,1965). The main clinical symptoms were diarrhoea and erosions and ulceration of the oral mucosa. Pyrexia, anorexia, salivation and nasal discharge usually also occurred, while some cases had skin lesions and were lame due to interdigital ulceration and inflammation of the coronary bands. The main pathological features were erosions, ulcerations, hyperaemia, haemorrhage and oedema of the mucosae of the alimentary tract with atrophic changes in lymphatic tissues. For some time, at least two separate diseases were thought to exist, but clinical and pathological similarities between the two syndromes were recognised and in an early review the name BVD-MD complex was introduced (Pritchard,1963). Viruses were isolated from animals suffering from BVD (Baker et al.,1954;



Gillespie et al.,1960) and MD (Underdahl et al.,1957) and serological tests showed that these and other isolates were antigenically related (Gillespie et al.,1961). Viruses from BVD and MD cases in U.S.A., West Germany, Scotland and England were also shown to be related (Kniazeff et al., 1961). For reasons of historical precedent the reference virus for the two syndromes was termed BVD virus (BVDV).

The name 'B' or 'Border disease' was proposed by Hughes et al.(1959) for a disease affecting young lambs of the Clun Forest and Kerry Hill breeds in counties on both sides of the England/Wales border. The syndrome was characterised by an increased amount of long, wavy, often abnormally pigmented, hair in the birth-coat of lambs a proportion of which showed nervous symptoms varying from a slightly defective gait to a violent tremor. Affected lambs were smaller, grew less well than normal and few survived beyond weaning. The major pathological finding was a deficiency of myelin throughout the central nervous system (CNS) of lambs with nervous symptoms. This finding led to the use of an alternative name, 'hypomyelinogenesis congenita', in a description of the neurological manifestations of the disease in lambs of mixed breeds (Markson et al.,1959).

In New Zealand a congenital chorea in lambs with hairy fleeces and reduced amounts of myelin in the CNS was first described in Romney crosses (Hartley and Kater,1962), and later in a wider variety of breeds (Manktelow et al.,1969). Affected lambs were described as 'hairy-shakers'.

The world-wide distribution of Border disease is shown by reports from North America (Osburn et al., 1972; Physick-Sheard et al., 1980), Ireland (Hamilton and Donnelly, 1970), Scotland (Dickinson and Barlow, 1967), Greece (Spais et al., 1975) and Australia (Acland et al., 1972).

The transmissible nature of BD was first shown by the inoculation of pregnant ewes with tissue homogenates of affected lambs and fetuses (Dickinson and Barlow, 1967; Shaw et al., 1967), and evidence for a viral aetiology came from transmission experiments using cell-free filtrates of tissue homogenates (Gardiner and Barlow, 1972).

#### CLINICAL DISEASES CAUSED BY OR ASSOCIATED WITH BVDV AND BDV Diseases in cattle and sheep

Since the original descriptions of the two syndromes no other diseases have been attributed to border disease virus which is, therefore, regarded solely as a cause of congenital disease with its most obvious clinical manifestations occurring at lambing time.

In contrast, BVDV has been associated with a number of other diseases. Serological surveys have shown that BVDV is widespread in cattle throughout the world (see Saurat et al., 1972). Ubiquity of the virus, often without related disease implies that many infections must be subclinical. However, as well as causing enteric disease, BVDV is an important cause of foetal disease when infections of susceptible pregnant cattle occur (Kahrs, 1973). Characteristically, both syndromes are

manifested by a wide range of symptoms from extremely severe to clinically inapparent.

The evidence for the role of BVDV in causing other diseases is not so conclusive, principally because of difficulties in reproducing the syndromes experimentally. Respiratory disease consisting of nasal discharges, persistent coughs and hyperaemia of the respiratory mucosae has been observed in some animals during field outbreaks of BVDV (Olafson et al.,1946; Dow et al.,1956). In a comprehensive investigation of viral causes of respiratory disease in young housed calves seroconversion to BVDV was associated significantly with outbreaks of disease, but no cytopathic BVDV isolates were made (Stott et al.,1980). Other workers have suggested that post-natal infection with BVDV predisposes animals to infection with other microorganisms and it is in this way that BVDV contributes to outbreaks of respiratory disease (Reggiardo and Kaeberle,1980; Roth and Kaeberle, 1982).

BVDV has been associated with infertility and early embryonic death in cattle (Archbald et al.,1979). In view of its ability to cause reproductive failure later in gestation this property would not be unexpected but experimental evidence is lacking. Indeed, seronegative cows infected by natural routes at the time of breeding had pregnancy rates comparable to uninfected controls (Whitmore et al.,1981). BVDV has been associated with an immune mediated ovaritis in infertile heifers but the significance of this finding must await further studies (Ssentongo et al.,1980).

It is, therefore, largely as causes of congenital infections in cattle and sheep that BVDV and BDV share common properties.

#### Cross-infections between cattle and sheep

The first cytopathic virus isolated from tissues of cattle with MD was shown to stimulate the formation of neutralising antibodies in sheep (Underdahl et al., 1957). This virus was not fully characterised but the first cytopathic reference strain, Oregon C24V, was successfully propagated by inoculating lamb fetuses in-utero (Taylor et al., 1963). The significance of these reports appears to have passed unnoticed. Neutralising antibody to BVDV was detected in ovine sera in U.S.A. and Australia (Seibold and Dougherty, 1967; St. George, 1971), but it was not until 1972 that sheep from flocks with BD were shown to produce neutralising antibody to BVDV (Acland et al., 1972; Hamilton and Timoney, 1972).

In the meantime, two strains of BVDV were shown to cause abortion and congenital defects when injected into pregnant sheep (Ward, 1971). The pathogenicity of BVDV isolates for the sheep foetus has since been demonstrated by other workers (Snowdon et al., 1975; Parsonson et al., 1979; Terlecki et al., 1980). The reverse is also true; material from BD lambs injected intramuscularly into pregnant heifers caused foetal death in 9 of 10 animals with all the dams developing antibodies to BVDV (Gibbons et al., 1974).

#### Diseases of other species

Pigs. Serological cross-reactions between HCV and BVDV continue to hamper HC control programmes in countries

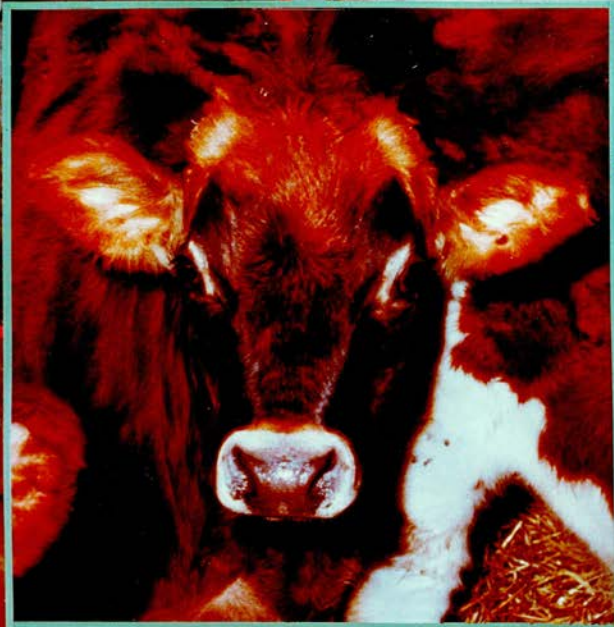
where that disease is endemic. Results from Australia and Ireland where virulent HC has never occurred have shown that antibodies to BVDV are readily detectable in some pig herds with no recognised disease problems (French and Snowdon, 1977; Lenihan and Collery, 1977). No natural outbreaks of disease in pigs due to pestiviruses more closely related to BVDV or BDV have been described but experimental infections have shown that infection can occur in a low percentage of piglets born to sows infected with BDV and BVDV in early gestation (Wrathall et al., 1978; Stewart et al., 1980).

Goats. Experimental infection of pregnant goats with BDV resulted in a high incidence of abortions and the birth of kids with tremor, hypomyelinogenesis and hypergliosis of the CNS (Barlow et al., 1975). A natural outbreak of border disease has been described in Norway (Loken et al., 1982), and in Australia a pestivirus was isolated from the pneumonic lung of a kid, but its role in the aetiology of the disease was unknown (Fraser et al., 1981).

Wildlife species. Outbreaks of MD and BVD-like diseases have been seen in wild deer in U.S.A. and wild ruminants in the Hanover zoo, but conclusive virological studies were not done (Karstad, 1981). Viruses related to reference strains of BVDV have been isolated from the spleens of roe deer and red deer but were probably not wholly responsible for the death of the animals (Romvary, 1965; Nettleton et al., 1980).

FIGURE 1.1      Species      of      British      domestic      animals  
susceptible      to      BVD      and      BD      viruses.  
Serological      surveys      have      shown      infection      to  
be      most      prevalent      among      cattle.





## PROPERTIES OF THE VIRUSES

Early studies showed BVDV to be a lipid-containing virus, approximately 40 nm in diameter containing infectious RNA (Hermodsson and Dinter, 1962; Diderholm and Dinter, 1966). Other BVDV isolates were susceptible to ether, chloroform, trypsin and heat and virions were shown to contain single-stranded RNA (Gillespie et al., 1963; Tanaka et al., 1968; Hafez and Liess, 1972). The presence of an envelope was confirmed by electron-microscope studies on partly purified virus preparations which also revealed a considerable size range of viral particles from 30 to more than 100nm (Ritchie and Fernelius, 1969). Other workers have described more homogeneous virus preparations with an average diameter 50-80 nm. Virions were pleomorphic, roughly spherical and covered with a compact membrane (Stott et al., 1974; Della-Porta, 1976). In some particles a distinctive 28nm core can be seen and beaded envelope structures have also been described (Horzinek, 1981).

Thin sections of BVDV-infected cell cultures have also detected viral particles 45-55 nm in diameter within characteristic vesicular structures (Chasey and Roeder, 1981). In a detailed study of viral morphogenesis it was concluded that a budding process was not involved in the development of BVDV (Ohmann and Bloch, 1982).

Low virus titres, fragility of the virus and contamination of purified preparations with cellular contaminants have all hampered detailed physico-chemical analysis of the virus. Three publications on the structure of viral RNA from the NADL strain of BVDV (BVDV-NADL) have



produced conflicting data. The major RNA component extracted from isopycnicly banded ( $^3\text{H}$ ) uridine labelled virus had a sedimentation coefficient of 38s but two minor components of 31s and 21s were also present. All three RNA species were susceptible to digestion with RNAase (Pritchett et al.,1975). In a second study one RNA species with a sedimentation coefficient of 40s was extracted from purified virus. This was also shown to be single stranded by its susceptibility to digestion with RNAase. In the same study two species of RNA were present in infected cells. One appeared identical to the viral RNA and the second RNA, which sedimented at 20s in sucrose gradients, was resistant to RNAase and was considered to be the replicative form of the virus RNA (Felmingham and Brown,1977). While further elucidation of the form, sedimentation characteristics and presence of heterogeneous RNA species in BVD virus populations is clearly required the studies have provided evidence that the molecular weight of viral RNA is between  $3.2 \times 10^6$  and  $3.6 \times 10^6$ , corresponding to 9.0 to 10.2 kilobases.

Conflicting results have also been reported on the structural components of BVDV-NADL. Early studies identified four virus-specific proteins having molecular weights of 110K, 93K, 70K and 23K (Pritchett and Zee,1975). Other workers have reported two major polypeptides, 144K and 138K, and two minor ones, 117K and 100K (Felmingham and Brown,1977), while in another study three structural proteins with molecular weights of 57K, 44K and 34K were described with the two larger proteins being glycosylated (Matthaeus,1979).

FIGURE 1.2      Thin sections of cells infected with BVD virus (Above) and BD virus (Below) reveal areas of modified rough endoplasmic reticulum (Arrows).

Magnification X 15,000

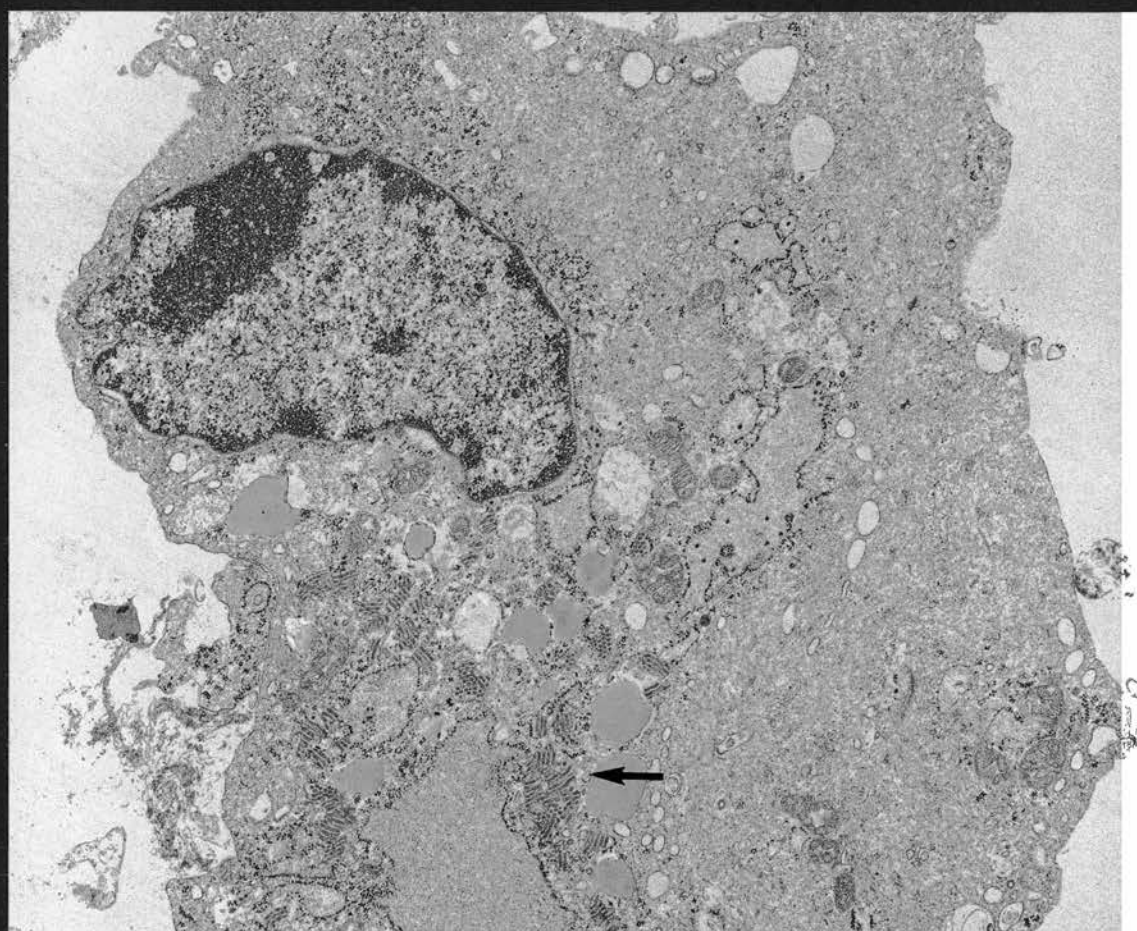
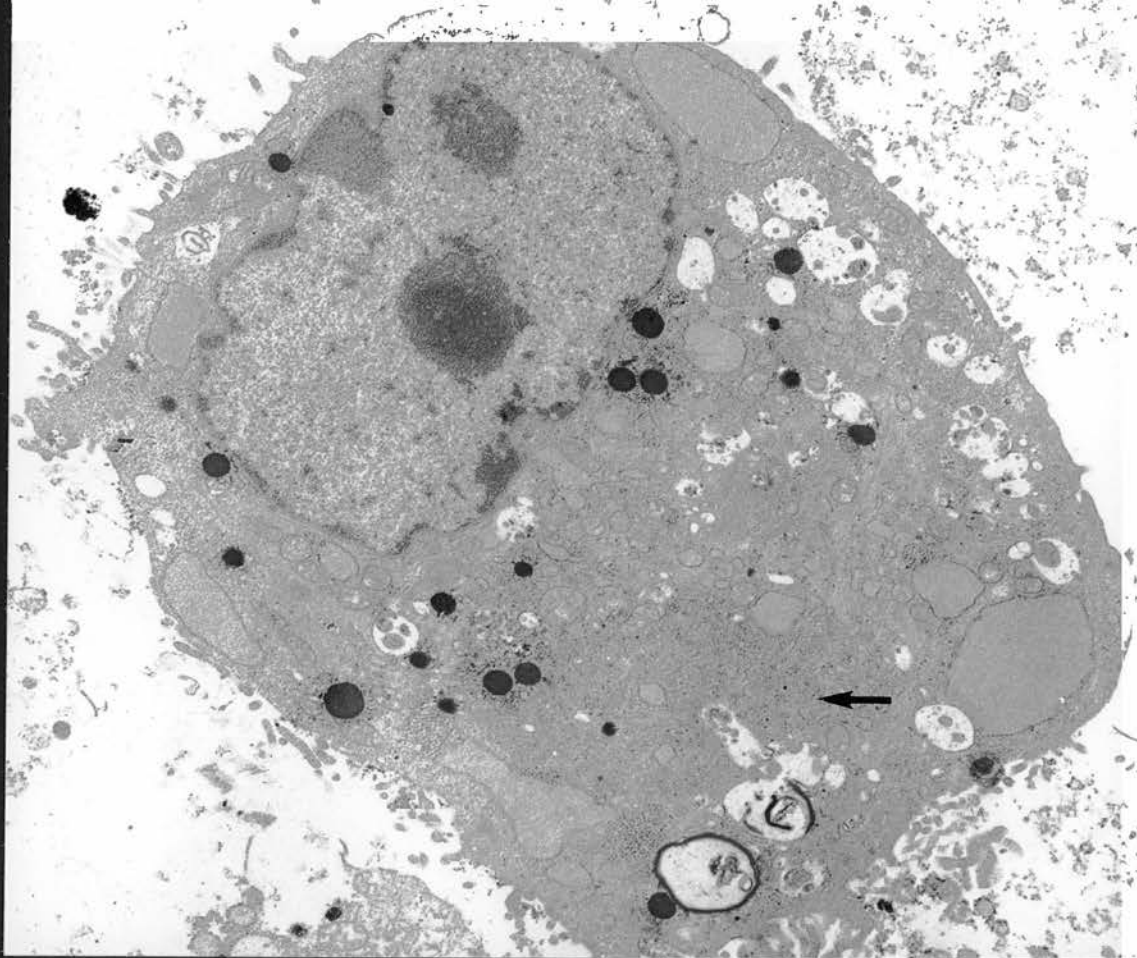


FIGURE 1.3     At higher magnification virus particles, 40-50 nm in diameter are visible closely associated with endoplasmic reticulum (Above), and in vesicles near the surface of the cell (Below). BVD virus.

Magnification X 60,000



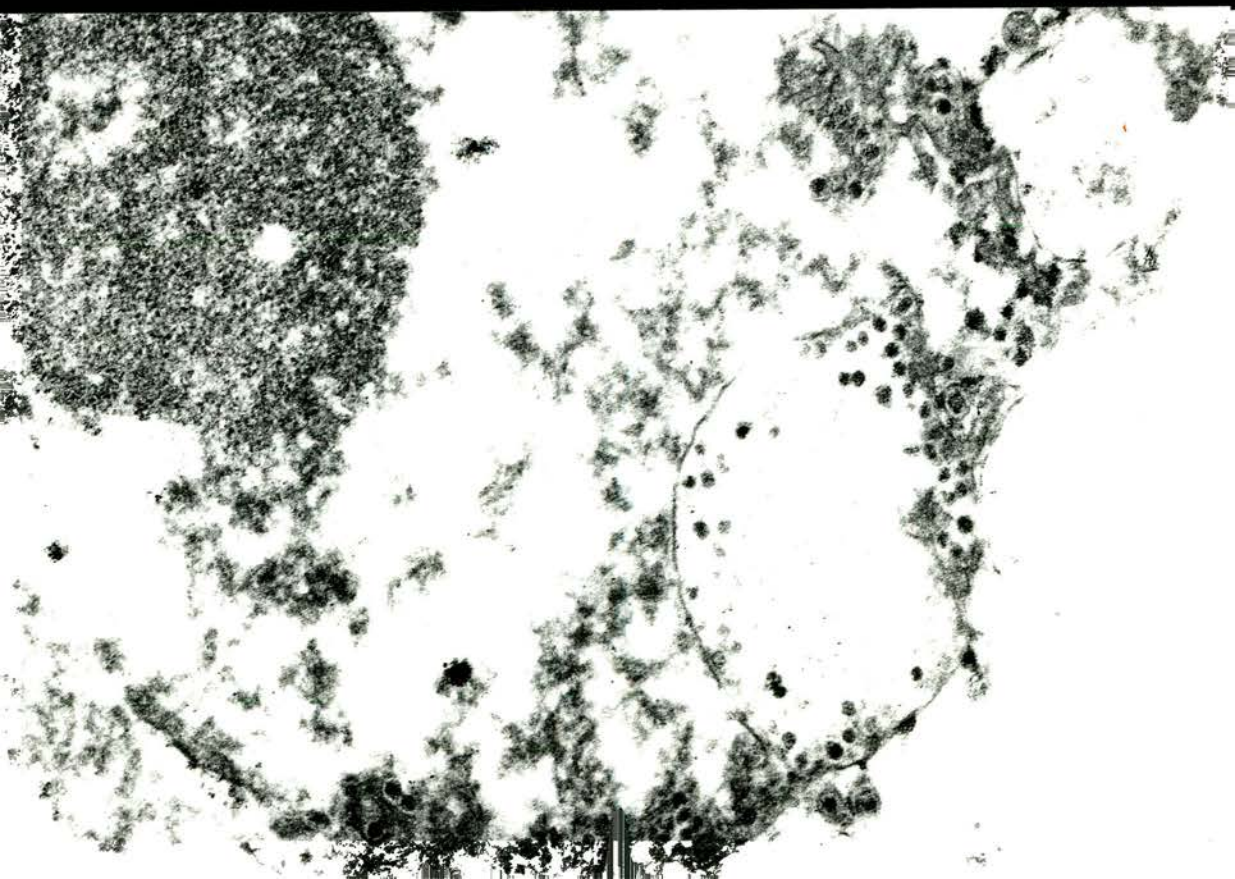
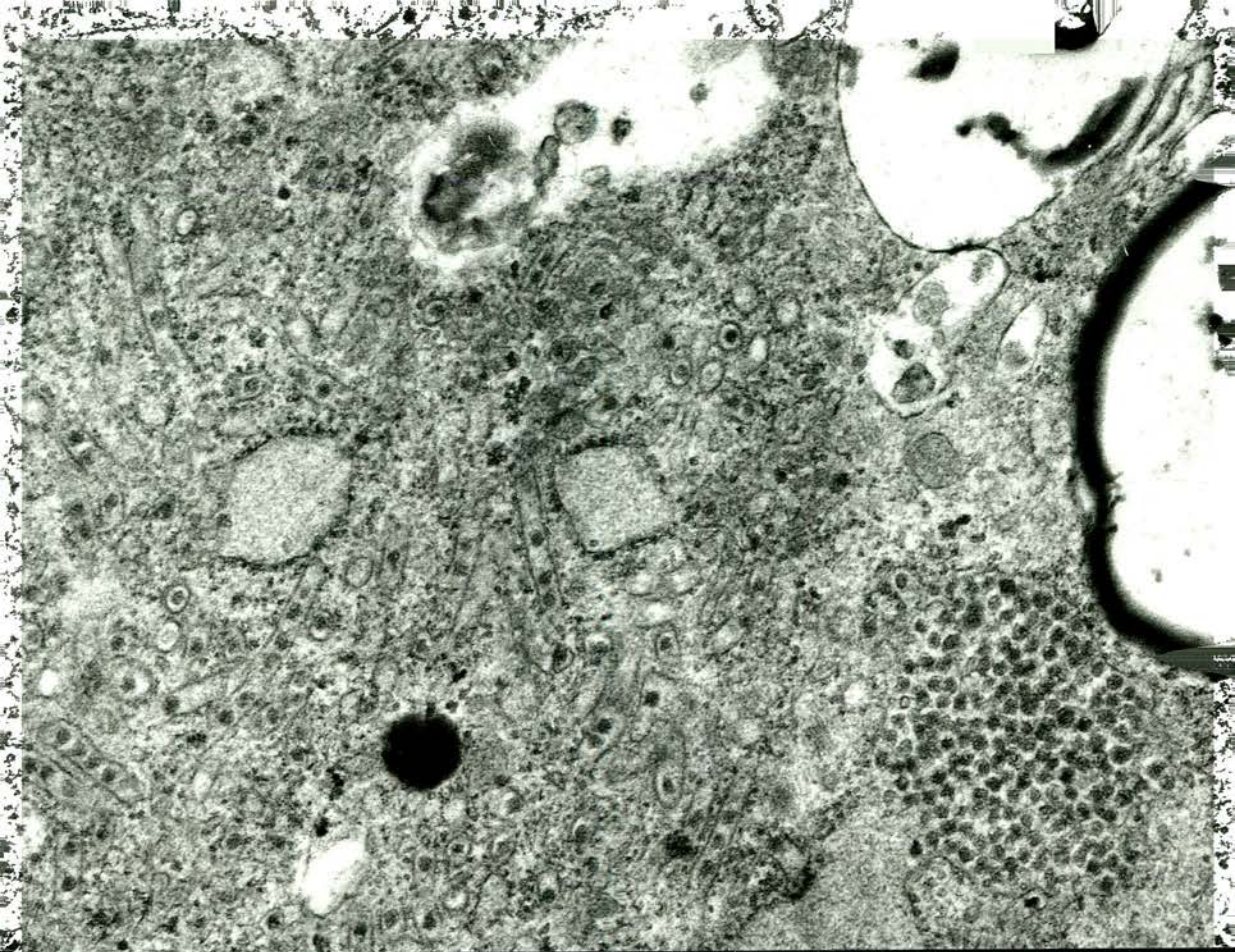
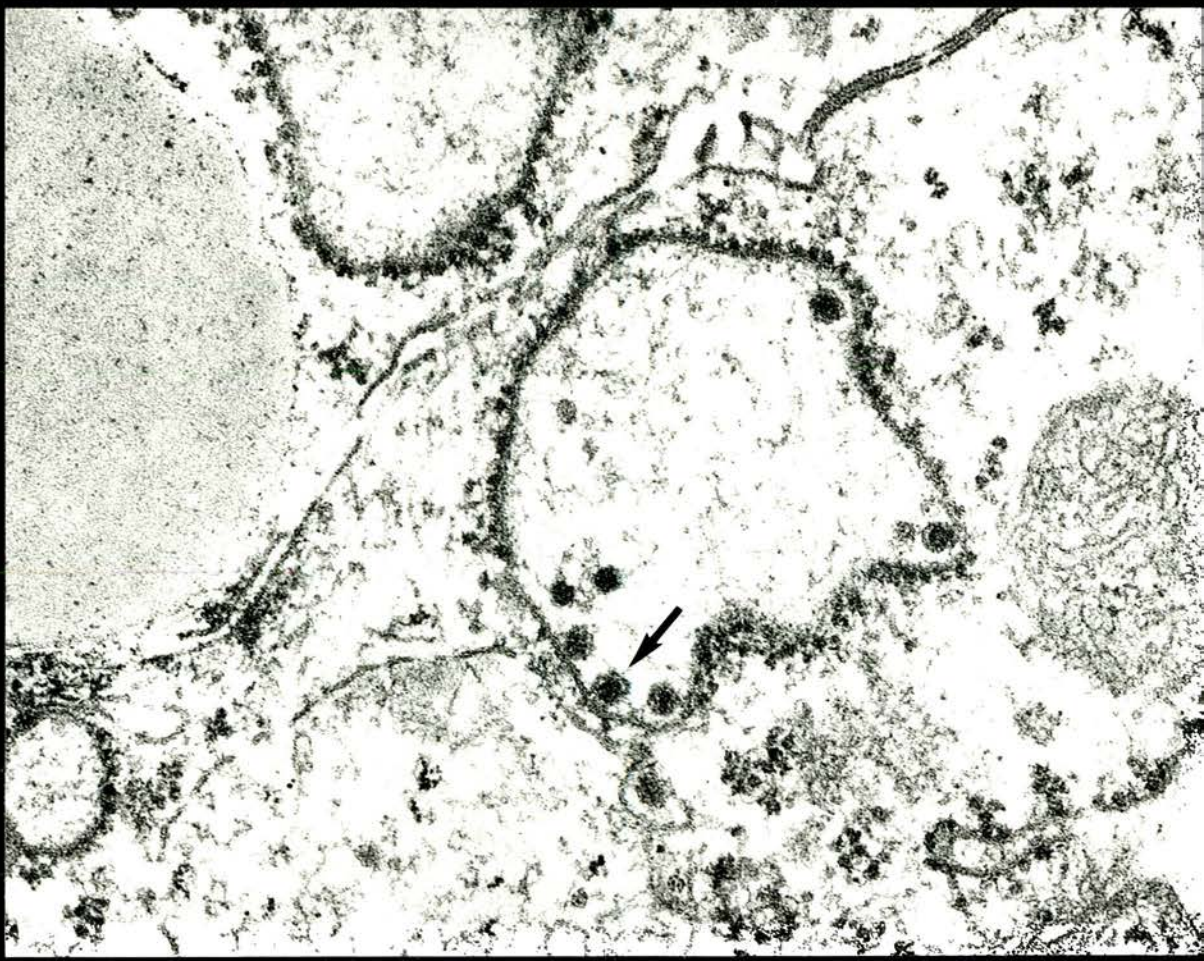
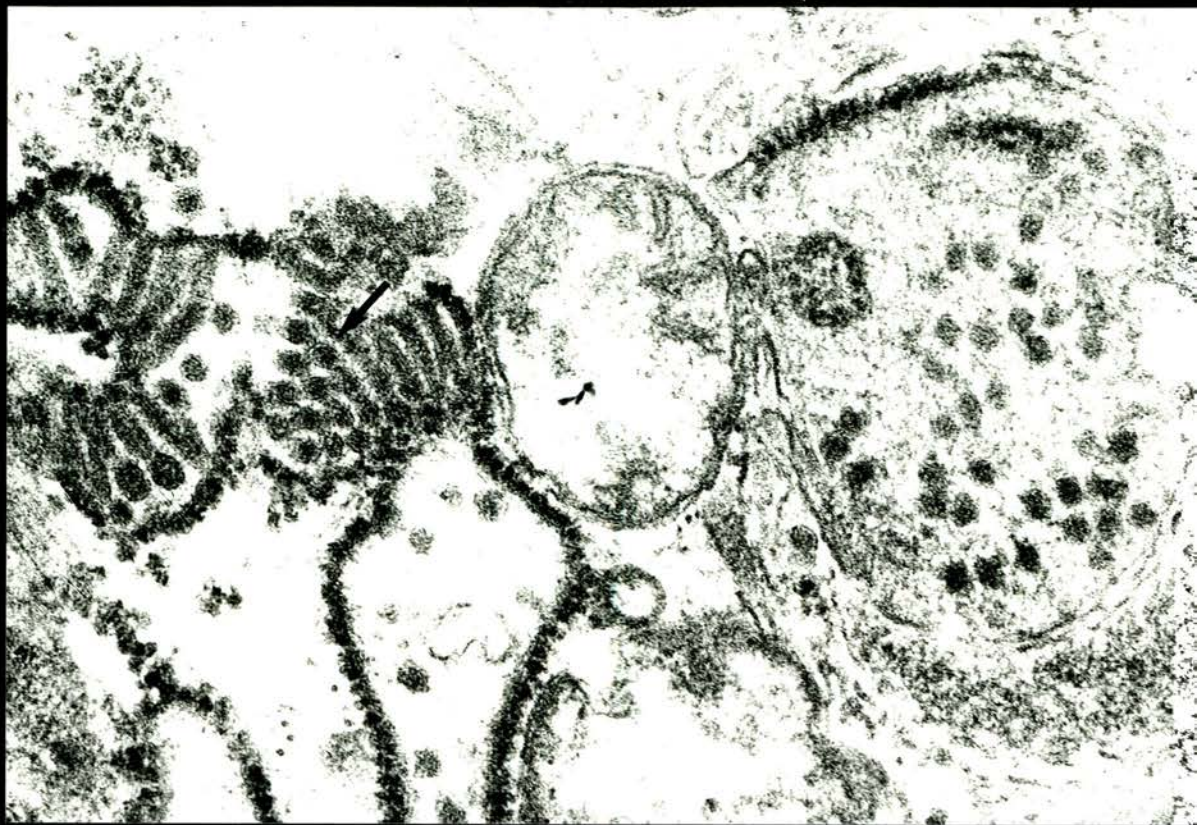


FIGURE 1.4      Pestiviruses have a very indistinct structure even in sharply focused prints. Although virus particles are not believed to 'bud' through cell membranes they may be seen closely associated with them (Arrows).  
BD virus.

Magnification X 90,000





There is much less controversy over the structure of BD viruses simply because no detailed studies have yet been done. Virus in infective nervous tissue was shown to be small, around 27nm, with an ether-soluble envelope. It was inactivated by heating to 60°C for 90 minutes (Gardiner et al., 1972). Studies on the cytopathic Moredun strain (BDV-M) and the non-cytopathic BD-2 strain have provided further evidence that infectious virus is small, sensitive to ether, chloroform, trypsin and heat (56°C for 30 minutes), and has RNA as its nucleic acid (Vantsis et al., 1976; Harkness and Vantsis, 1982). Comparative centrifugation analysis of BD-2 virus with BVBD-NADL showed that both had buoyant densities of 1.105 g./ml. in Urografin gradients (Harkness and Vantsis, 1982). Other values for BVDV-NADL in caesium chloride gradients have been reported as 1.14 g./ml. and 1.15 g./ml. (Hafez and Liess, 1972; Della-Porta, 1976). In sucrose gradients BDV-M had a buoyant density of 1.115 g./ml. (Vantsis et al., 1976). This value is also similar to those of 1.092 g./ml. and 1.11 g./ml. obtained for BVDV-NADL under comparable conditions. (Parks et al., 1972; Stott et al., 1974).

BVDV and BDV, therefore, both have physico-chemical properties consistent with their classification in the family Togaviridae. In sucrose density gradients the sedimentation coefficients and buoyant density of nine BVDV strains, two BDV strains and six HCV strains were very similar and these viruses were distinguishable from other representative members of the togavirus family. It was



concluded that BVDV, BDV and HCV form a structurally homogeneous group within the Togaviridae (Laude,1979).

#### ANTIGENIC RELATIONSHIPS

Within the Togaviridae. The majority of togaviruses depend on biological transmission by arthropods for their survival. This is true of all alphaviruses and most flaviviruses, the two major antigenically distinguishable genera within the family. Antigenically distinct from these two groups are those togaviruses which do not have an arthropod vector, the so-called "non-arthropod-borne" or nonarbo togaviruses (Horzinek,1973). Within the nonarbo togaviruses two genera have been distinguished; Rubivirus with its sole representative member rubella virus, and Pestivirus within which BVDV, BDV and HCV are grouped. The pestiviruses are serologically related but unrelated to rubella virus (Porterfield et al.,1978). Between the pestiviruses the basis for the formation of the pestivirus genus came from the discovery of a serological relationship between viral antigens present in tissues of animals suffering from swine fever(HC) and mucosal disease(BVD) using an agar gel diffusion test (Darbyshire,1960; Darbyshire,1962). This relationship has been studied intensively using a variety of serological methods, largely to explore the feasibility of exploiting BVDV vaccines to protect pigs against HC. Further work on the common precipitation lines in immunodiffusion tests (IDT) using infectious tissue culture fluids have shown that the precipitating antigens are "soluble" antigens (SA)

separable from infectious viral particles by ultracentrifugation and ultrafiltration (Gutekunst and Malmquist, 1963). Immuno-electrophoresis (IEP) has confirmed a reaction of identity between "soluble" HCV and BVDV antigens (Matthaeus and Van Aert, 1971; Dalsgaard, 1976). The common pestivirus antigen is a glycoprotein (Dalsgaard and Overby, 1977) but its relationship to virus structure remains unknown. Both IDT and IEP have shown that the common SA of HCV and BVDV are not viral structural proteins (Matthaeus, 1977; 1980). The immunological relationship between BDV, BVDV and HCV has been confirmed by ID tests with no analysis of the precipitating proteins (Plant et al., 1973).

An antigenic relationship between HCV and BVDV has been established by complement fixation (CF) tests (Eskildsen and Overby, 1976) and CF tests have been used to measure the formation of "soluble" BVDV antigen in infected cells. The SA was detectable intracellularly prior to the appearance of infectious virus and was released from cells at the same time as the virus (Gutekunst and Malmquist, 1965). When sheep and goats were inoculated with tissues from cases of BD, CF antibodies to BVD antigens were found in the sera (Huck et al., 1975).

Immunofluorescence (IF) tests have also been used to demonstrate the relationship between HCV, BVDV and BDV. For example HCV antisera have been used to detect BDV (Harkness et al., 1977; Terpstra, 1978). Some sera, notably those raised against BVDV which lack neutralising antibody against HCV still react strongly with HCV in IF tests

(Horzinek,1981). This shows that the determinants of antigen detected by IF are different from those functioning in virus neutralisation. Further evidence to support this has come from studies on the time of appearance of fluorescence in both HCV and BVDV infected cells. Intranuclear fluorescence has been detected in a few BVDV infected cells but diffuse intracytoplasmic fluorescence originating close to the nuclear membrane is usually first seen in BVDV and HCV infected cells as early as 4-6 hours post-infection. After reaching maximum intensity and extent between 20 and 60 hours fluorescence gradually fades (Mengeling and Drake,1969; Fernelius,1969). Both these reports provide evidence that the appearance of specific fluorescence coincides with the appearance of SA before the production of infectious virus.

The antigenic relationship between the three pestiviruses as detected by ID, IEP, CF and IF tests, therefore appears to be largely dependent on a common SA. This is a virus-induced non-structural glycoprotein, which from its early appearance in infected cells is probably a precursor of a viral component. SA prepared from BVDV injected into calves and pigs had some protective function apparently by priming the immune system. Calves showed a rapid development of neutralising antibody following challenge (Gutekunst and Malmquist,1964). Pigs were protected against challenge with virulent HC and showed an accelerated rate of HCV antibody production following exposure (Volenec et al.,1972).

The relationship between pestiviruses has also been established by the detection of cross-neutralising antibodies. It has become clear, though, that the degree of cross-neutralisation between pestiviruses is very variable, and depends on the strains used. The relationship between HCV and BVDV is weak with only some hyperimmune sera against HCV neutralising BVDV (Dinter, 1963; Corthier, 1976). In pigs, some strains of BVDV produce antibodies but the level of cross-neutralisation against HCV depends on the strain used for immunisation (Snowdon and French, 1968; Stewart et al., 1971). In one study one strain of BVDV was capable of protecting some pigs challenged with HCV whereas those strains less closely related in serum neutralisation (SN) tests failed to protect. In the same study calves injected with HCV were resistant to challenge with BVDV regardless of the challenge virus used. It was also noted that homologous antibody production occurred earlier, following inoculation of HC virus, in pigs previously injected with BVDV than in the control pigs. The same findings with regard to the antibody to BVDV were obtained in calves previously inoculated with HCV (Castrucci et al., 1970).

Cross-neutralisation has also been demonstrated between BDV, BVDV and HCV (Osburn et al., 1973). Again strain differences appear to be important. In pigs, higher homologous SN titres were produced by the Moredun strain of BDV than by the BD-2 strain. Pigs receiving Moredun virus survived HCV challenge while those injected with BD-2 virus succumbed (Laude and Gelfi, 1979). Cross-neutralisation

between BDV-M and BVDV-NADL has been demonstrated. In sheep injected with BDV, SN antibodies developed earlier and to higher titres to the homologous virus than to BVDV-NADL (Vantsis et al.,1980).

Within BVDV strains. Considerable serological variation has been detected in cross-neutralisation tests between BVDV strains (Castrucci et al.,1968; Hafez and Liess, 1972). Demonstrable strain differences led to a classification into 3 serotypes represented by the NADL strain, the Oregon C24V strain and three non-cytopathic strains. These serotypes were also related to the type of Cytopathic effect (CPE) produced in bovine embryonic kidney (BEK) cells. Changed viruses, i.e. "biotypes" produced by passage in cells or rabbits, were concomitantly converted to the serotypes of the virus they biotypically resembled (Fernelius et al.,1971). The use of anti-NADL and anti-Oregon C24V sera against 17 BVDV isolates failed to produce any evidence for the existence of different serotypes, but some significant serological differences between the virus strains indicated a wide antigenic heterogeneity (Corthier and Aynaud, 1973). A study of three serologically heterologous strains of BVDV in reciprocal cross-immunity tests in calves showed good protection. Each of the 3 strains provoked the production of antibody to the heterologous as well as to the homologous strain (Castrucci et al.,1975).

Within BDV strains. Border disease virus isolates have been differentiated on the basis of their serological relationship to BVD-NADL (Vantsis et al.,1979). The

Moredun strain of BD virus is different serologically from BVDV-NADL. In a natural outbreak of BD, ewes developed higher SN titres to BVDV-NADL than to BDV-M. During their next pregnancy ewes were susceptible to challenge with BDV-M but not to the homologous BD virus (Vantsis et al., 1980).

#### VIRUS CULTIVATION

##### Host range in laboratory animals and chick embryos.

Attempts to propagate BVDV in embryonated hens' eggs, mice, unweaned mice, rat foetuses, guinea-pigs, hamsters, ferrets and pigeons were all unsuccessful (Olafson and Rickard, 1947; Baker et al., 1954; Huck, 1957; Taylor et al., 1963). Of all laboratory animals only rabbits have been used for successful passage of the virus (Baker et al., 1954; Fernelius et al., 1969).

Host range in tissue culture. Virtually all researchers into BVDV have used virus propagated in cells cultured from trypsinised tissues. Primary cells and some cell lines of cattle, sheep and pig tissues have been shown to be susceptible to both BVD and BD viruses. The cytopathic NADL and C24V strains of BVDV have been adapted to established cell lines of hamster kidney (HAK) and human cells resembling the HELA line (ERK-10), but both became non-cytopathic and were detectable only by immunofluorescence (IF) tests (Fernelius et al., 1969). Truitt and Shechmeister (1973) reported adaptation of BVDV-NADL to cells of a canine kidney (CK) line in which a CPE was produced 96 hours after infection. In the absence

of confirmation of this result it would appear possible that the CK line may have been a calf kidney cell line. In the same report it was shown that BVDV replicated in bovine macrophage and lymphocytes cultivated in-vitro.

For studying BVDV, primary or low pass bovine cells particularly kidney cells have been most widely used (Underdahl et al., 1957; Gillespie et al., 1963; Mills et al., 1965). Other workers have preferred bovine testis cells (Van Bekkum and Straver, 1964) or bovine embryonic spleen cultures (Malmquist, 1968). In a study of plaque formation by BVDV-NADL similar sized plaques were formed in kidney, lung and testis cultures derived from bovine foetuses while smaller plaques were formed in lamb testis cultures (Singh, 1969a). In contrast to this, one isolate of BDV has been shown to replicate well in foetal lamb kidney (FLK) cells but poorly in BEK cells (Vantsis et al., 1976). Other BDV strains have been grown in sheep choroid plexus cells (Niemi et al., 1982; Potts et al., 1982), but bovine testis cells were used for the replication of an Australian isolate (Plant et al., 1976).

The use of bovine cultures has been plagued by the adventitious contamination of cells with non-cytopathic BVDV. The virus has been shown to originate from the cells themselves and from the foetal calf serum used to supplement the cell growth medium. Cells shown to be contaminated have been BEK (13 of 133 foetuses tested), calf testis (1 of 14 tested), calf kidney (10 of 63 tested), and bovine foetal lung (1 of 37 tested) (Smithies and Modderman, 1975; Nuttall et al., 1977; Rossi et



al.,1980). BVDV contamination rates of commercially available foetal calf serum (FCS) have been variously reported as 17% (1 of 6 batches), 75% (14 of 19 batches), 100% (5 of 5 batches) and 62 % (13 of 21 batches) (King and Harkness,1975; Smithies and Modderman,1975; Nuttall et al.,1977; Rossi et al.,1980). Heat inactivation at 56°C for 30 minutes, and irradiation of FCS have both been shown to reduce greatly but not totally eliminate BVDV contamination arising from this source (Nuttall et al.,1977; Rossi et al.,1980). Given this apparent widespread contamination of bovine tissue culture systems with non-cytopathic BVDV it is possible that many experiments conducted before this problem was recognised were compromised to some extent.

One way round the contamination problem would be to use cell lines shown to be free of BVDV grown in medium supplemented with virus-free serum. There are, however, few reports of successful virus growth using such a system, the majority of workers apparently preferring to use cells with a low passage history. The reasons for this are not immediately apparent from the literature, although passage of BVDV virus in a PK-15 cell line resulted in a change in the CPE when the virus was later grown in primary BEK cells (Fernelius et al.,1969a). The most consistent use of a cell line has been by workers at the National Animal Diseases Laboratory, Ames, Iowa, U.S.A. who have used a bovine turbinate (BT) line for growing BVDV (McClurkin et al.,1974). A foetal lamb muscle cell line has been shown



to be useful for the propagation and assay of both BVDV and BDV (Laude and Gelfi,1979). As well as being used for the growth of BVDV, the PK-15 line has been used for culturing a non-cytopathic isolate of BDV (Potts et al.,1982).

Cytopathogenicity. Both cytopathic (CP) and non-cytopathic (NCP) strains of BVDV and BDV have been described. In a comprehensive list of thirty strains of BVDV isolated between 1954 and 1969, nineteen were CP and 11 NCP (Saurat et al.,1972). Expression of cytopathogenicity depends on inherent properties of the virus strain, but can also be influenced by the conditions in which it is cultured. The first reference strain of BVDV isolated was designated VD New York 1 (NY-1) (Baker et al.,1954). This was later shown to replicate well in bovine skin, muscle and embryonic kidney cells without producing a CPE (Lee and Gillespie,1957). No CPE was produced when the virus was cultured in a range of primary bovine cells as well as in cell lines of bovine, ovine, porcine and caprine kidney, and human cervix (HeLa), liver (Chang) and intestine (Henle) (Pritchard,1963). The two other major reference strains of BVDV, Oregon C24V (Gillespie et al.,1960) and BVDV-NADL (Gutekunst and Malmquist,1963) are both cytopathic in BEK cells but the type of CPE produced is different. CPE is slow to develop in C24V infected cultures, multiple intracytoplasmic vacuolation occurs, nuclei become pyknotic and a low percentage of cells round up and detach from the glass. A much more rapid and complete CPE is caused by the NADL-MD strain. Cells develop pyknotic nuclei and round up and detach from the

glass before intracytoplasmic vacuolation develops (Fernelius and Ritchie, 1966).

The CPE in BEK cells of BVDV-C24V and BVDV-NADL has been shown to change following passage in different cell systems. After passage in a PK-15 cell line BVDV-C24V was NCP, while BVDV-NADL retained its cytopathogenicity. After adaptation to rabbits, though, the cytopathogenicity of NADL resembled that of C24V. Change in the type of CPE was termed "biotypic conversion" (Fernelius et al., 1969a; 1969b). In the discussion of the second of these papers there are reports of a non-cytopathic strain which is CP for certain batches of BEK cells. This and two other named strains with the same capability were described as being of intermediate virulence. The belief was expressed that there are BVDV strains having high, intermediate and low cytopathogenicity. This was confirmed by French and Snowdon (1964) who isolated virus of all three types from their clinical cases.

Further evidence that the cytopathogenicity of pestiviruses can vary according to the culture conditions has come from studies with BDV. The majority of BDV isolates have been non-cytopathic in vitro, and have been detected by interference tests (Hadjisavvas et al., 1975) or immunofluorescent tests (Harkness et al., 1977; Terpstra, 1978). There are three reports of the isolation of cytopathic agents. In an Australian study of ovine perinatal mortality, 4 viruses were isolated which were serologically related to the C24V strain of BVDV and which were cytopathic for bovine testis cells (Hore et al., 1973).

One of these viruses, strain HE 18, caused abortion and was shown to infect fetuses when injected intravenously into pregnant ewes (French et al., 1974). Vantsis et al., (1976) isolated a cytopathic virus (BDV-M) from homogenised brains of experimentally produced BD lambs by inoculating FLK cultures. Cytopathic isolates have also been recovered in sheep choroid plexus cells from nasal and vaginal swabs from post-partum ewes (Niemi et al., 1982). Interestingly, cytopathic isolates from these last two laboratories produced little or no CPE in calf kidney or primary BEK cells. Similarly BDV isolated and passed in calf testis cells was NCP in these cells but was cytopathic in a foetal lamb muscle cell line (Laude and Gelfi, 1979).

While the cytopathogenicity of some BVDV and BDV isolates can vary depending on the cell system used other isolates are genuinely non-cytopathic. Comparative studies of the growth of CP and NCP strains of BVDV showed no difference in the kinetics of replication but the CP virus produced 58 times more infectious virus per cell (Nuttall, 1980). A single study on the mechanism of persistence by NCP virus concluded that persistence was not the result of the induction of defective interfering particles or the selection of temperature sensitive mutants but was the result of a stable phenotypic property of the virus (Coria and McClurkin, 1981).

#### PATHOGENESIS OF DISEASES IN CATTLE AND SHEEP

Experimental infections to study the pathogenesis of disease have, naturally, attempted to reproduce the syndromes seen in field outbreaks. Studies on enteric

disease have yielded much information on the response of young cattle to BVDV while the equivalent response of sheep to BDV is virtually unknown, since investigations have focused on pregnant animals. Except where stated, experimental infection of sheep refers to infection with BDV, and that of cattle with BVDV.

Foetal disease following the exposure of susceptible dams is, almost certainly, the most serious consequence of pestivirus infections in cattle as well as sheep. Congenital infection plays a central role in the pathogenesis and epidemiology of disease in both species and will be considered first.

#### CONGENITAL INFECTION

Experimental infections of pregnant cattle and sheep have shown that pestiviruses can cause a wide range of foetal disease varying from foetal death and abortion to the birth of live apparently normal offspring, some of which may be persistently infected with virus and yet remain healthy for years.

The maternal infection is usually subclinical or mild. In sheep, mild fever six to eleven days after inoculation and transient neutropaenias detected ten days after infection have been recorded (Shaw et al., 1967; Vantsis et al., 1979). In cattle, pyrexia and leucopaenia are common usually without malaise. Occasional animals show anorexia, diarrhoea and nasal, lachrymal and vaginal discharges (Kendrick, 1971; Brown et al., 1979).

The most important route of foetal infection is transplacental. Exposure of susceptible pregnant dams

results in viraemia with spread of virus to the placenta (French et al.,1974; Snowdon et al.,1975). Transmission to the foetus has been achieved by infected semen in sheep, but under natural conditions this is unlikely to be a major route of infection (Gardiner and Barlow,1981).

Replication of the virus in the placenta has been studied more in sheep than in cattle. Since both animals have similar forms of placentation, epitheliochorial in which 6 tissue layers separate maternal and foetal blood, it is likely that similar mechanisms occur in both species. A placentitis has been detected as early as 10 days after infection in pregnant ewes. Lesions developed in the endothelium of the maternal capillaries resulting in occlusion of the vessels and focal or diffuse necrosis of the caruncular septa. The lesion spread so that, ultimately, necrotic septal tissue dissected the foeto-maternal union. Bleeding from maternal capillaries accentuated the separation of the union between foetus and mother, with blood and necrotic debris being ingested by the trophoblast. The placentitis may be severe enough to contribute to the early foetal death and abortion which occur in some cases, but if the pregnancy is sustained the placentitis heals in about 25 days (Barlow,1972).

In another study BDV grew to high titres in the placentome of sheep without producing lesions, and yet still crossed to the foetus and caused abortion (French et al.,1974). There is some evidence from both sheep and cattle that virus does not cross to the foetus in the first month of pregnancy (Manktelow et al.,1969; Whitmore et

al.,1981)). A possible explanation for this could be that implantation and the development of close contact between maternal villi and the trophoblast is essential for virus to infect the foetus.

Once the placenta has been penetrated the virus is protected from the maternal immune response which has occurred following infection (Gardiner,1982). The foetus and virus are on their own. The ultimate outcome of this particular relationship depends on several factors, the major one of which is the stage of foetal development at which infection occurs. The most serious consequences occur following exposure during early pregnancy. Other factors include the strain and dose of the virus, the breed of the foetus and its ability to repair damage.

A critical factor in determining the distribution and persistence of virus, which, in turn, influences the extent of foetal damage is the age at which the foetus gains immunological competence. The ovine foetus can first respond to an antigenic stimulus between 64 and 82 days of its approximately 150 day gestation period (Fahey and Morris, 1978). In cattle, which have a gestation period of approximately 280 days, a specific immune response to BVDV has been detected in foetuses inoculated at 135-150 days gestation (Ohmann et al.,1982). Development of foetal immune competence, therefore, starts around mid-gestation in both species.

Knowledge of the foetal response to pestivirus infection has been derived predominantly from experiments in which the foetus has been infected via the dam. The



speed with which the virus crosses the placenta is unknown so that the actual timing of foetal infections may be only approximate. Nevertheless, some clear findings have emerged.

There is considerable evidence that foetal infection in cattle after 6 months gestation rarely causes disease and provokes a specific immune response similar to that in post-natal life so that calves are born with neutralising antibody (Ward et al.,1969; Braun et al.,1973; Brown et al.,1979). In sheep, infection of the foetus after 80 days results in the birth of clinically normal lambs. These lambs have a characteristic nodular periarteritis, which was suggestive of a cell-mediated allergic reaction (Zakarian et al.,1976). Neutralising antibody to BD was detected in precolostral sera from 20 of 39 lambs whose dams had been infected at 90 days (Gardiner et al.,1980).

There is, thus, good evidence in both species that foetal infection in the last third of pregnancy results in the birth of normal offspring possessing specific neutralising antibody to their infecting agent. Viral antigen has been detected in association with the lesions of periarteritis for up to 6 months after birth but it is not known if such lambs excrete virus (Gardiner et al.,1980). This would seem unlikely. Calves experimentally infected as fetuses late in pregnancy and born with specific neutralising antibody failed to infect susceptible calves with which they were kept for the first 4 months of life (Ward,1969).

The outcome of foetal exposure to virus at other stages of gestation is less predictable. Understandably, more information is available from sheep experiments than from cattle infections. The production of clinically affected "hairy-shaker" lambs has been achieved most consistently by infecting ewes between 50 and 63 days gestation, that is, before the onset of immune competence. In such lambs viral antigen is widespread (Terpstra,1978). There are, however, few histopathological changes in the viscera, although reduced thymus weight and poorly developed lymphatic nodules and a scarcity of lymphocytes in the spleen have been noted (Richardson,1982). Typically there is no evidence of any inflammatory reaction. The principal pathological finding, myelin deficiency in the CNS, is thought to result from direct viral action on oligodendrocyte precursors, leaving a deficit of mature myelin-forming cells at critical stages of CNS development (Barlow,1982). Precolostral sera from "hairy-shaker" lambs do not contain specific neutralising antibodies against pestiviruses. (Vantsis et al.,1979). Such lambs are tolerant to the virus and have a persistent infection usually for life (Plant et al.,1977).

Following some experimental BD infections at 50-55 days gestation, inflammatory lesions heavily infiltrated with macrophages and mononuclear cells, and liquefactive necrosis have been seen in the granulo-prival layers of the CNS. These lesions were seen within 21 days of infection in sacrificed foetuses and were considered to be the cause of severe brain malformations seen in lambs surviving to

term. The malformations included hydranencephaly, porencephaly and cerebellar hypoplasia, and some lambs also had arthrogryposis. Affected lambs had none of the clinical or pathological stigmata of classical BD (Clarke and Osburn, 1978; Barlow, 1980). The reasons for the development of this "alternative pathology" (AP) have not been fully elucidated. A review of the conditions under which it had occurred showed that the nature of the agent, and the breed and immune status of the ewe were important factors (Barlow, 1980). There is also an altered immune responsiveness of the foetus since the majority of lambs with AP are born with specific neutralising antibody to BDV (Gardiner, 1982). This precocious immune response by the foetus would certainly explain the inflammatory and necrotic lesions of AP.

Analogous findings have been reported in cattle. Bovine foetuses exposed to some strains of BVDV between 3 and 6 months gestation have shown CNS and ocular lesions at birth. The CNS lesions were principally cerebellar degeneration and hypoplasia (Ward, 1969; Brown et al., 1973). The ocular lesions have included retinal atrophy, optic neuritis, cataract and microphthalmia with retinal dysplasia (Bistner et al., 1970). The cerebellar lesions were considered too severe to have been caused by direct cell destruction due to the virus. They were interpreted to be a consequence of vasculitis which caused folial oedema with subsequent severe cerebellar destruction and it has been proposed that the vasculitis may have an immune component (Brown et al., 1974). Indirect evidence to

support this comes from the regular finding that virus cannot be detected from such animals and they have neutralising antibodies to BVDV at birth. Occasionally both virus and antibody have been detected in the same animal but, in general, the presence of SN antibody was associated with a failure to recover virus from foetal tissues (Badman et al.,1981).

The outcome of early foetal infection clearly depends on a dynamic interplay between viral and host properties. The unpredictability of the outcome was demonstrated in one experiment in which a wide range of possible sequelae occurred. Fifteen susceptible pregnant heifers received the same inoculum of a mixture of field isolates of BVDV at 100 days gestation. This resulted in 5 foetal deaths and the birth of 10 live calves. All calves showed evidence of intra-uterine growth retardation and 3 were affected with clinical nervous disease. Two of these 3 had brain malformations. They were born with specific SN antibody to the virus and were virus free. Virus was recovered from the remaining 8 calves which were antibody free. These 8 calves were considered to be immunotolerant to BVDV (Done et al.,1980).

In both cattle and sheep, therefore, there is evidence of a very complex interaction between the infecting pestivirus, the dam and the foetus. Virus strains vary in their antigenicity and pathogenic potential. The dam can prevent or modify the virus attack on the foetus. The foetus, depending on its stage of development, can react immunologically and rid itself of

the virus, but sometimes at the cost of causing severe malformations. Early foetal infection without an immune response can cause foetal death and abortion but the low pathogenicity of some viral strains ensure the survival of offspring which are persistently infected with, and apparently immunotolerant to their infecting virus. These animals are of immense importance in the epizootiology of BVD and BD.

#### PERSISTENT INFECTION IN POST-NATAL LIFE

Persistent infections often continue into post-natal life, and are characterised by a generalised infection in the host, which constantly sheds virus into the environment. The existence of cattle persistently infected with BVDV has been recognised for many years but persistence was generally associated with unthriftiness (Malmquist,1968). More recently it has been shown that persistent infections with BVDV can occur in apparently healthy animals (Coria and McClurkin,1978b). Similarly, both "hairy-shaker" and apparently normal lambs have been shown to be persistent excretors of BDV (Terpstra,1981).

Persistent infections have been established experimentally by both cytopathic and non-cytopathic strains of virus grown in tissue culture. Cattle given BVDV which was cytopathic in-vitro produced persistently infected calves from which only non-cytopathic virus could be recovered (Done et al,1980). When the same inoculum was used in pregnant ewes virus recovered from infected lambs was again found to be non-cytopathic (Terlecki et al.,1980). This suggests some alteration to the virus

within the host and could explain why Kendrick (1971), who only looked for cytopathic virus, did not detect virus in calves born to dams infected in early gestation. Non-cytopathic BDV has also been shown to establish persistent infections in lambs (Terpstra,1981).

Persistently infected cattle and sheep are viraemic and have no, or low, levels of anti-pestivirus neutralising antibodies. They readily yield non-cytopathic virus from blood and bodily secretions and appear to be immunotolerant to the virus infecting them. Normal levels of serum protein and immunoglobulins and the ability of the animals to produce antibodies to other agents confirm that persistently infected animals can have a normal humoral responsiveness in spite of their specific immune tolerance (Coria and McClurkin,1978b; Terpstra,1981). Development of antipestivirus antibodies by some persistently infected sheep in later life also shows that the immune tolerance may not be absolute (Terpstra,1978; Westbury et al.,1979).

Persistently infected animals often have reduced fertility. In one report cows had reduced conception rates, aborted or gave birth to persistently infected calves. A bull had poor quality sperm which contained virus but, when seropositive cows were bred normal virus-free calves were born. When seronegative heifers were bred, successful conception and pregnancy resulting in normal calves occurred only following seroconversion (McClurkin et al.,1979). Virus-containing semen from a persistently infected ram produced infected offspring (Gardiner and Barlow,1981), and persistently infected ewes



have produced infected lambs in successive pregnancies (Westbury et al.,1979; Barlow et al.,1980). Vertical virus transmission by persistently infected animals, particularly females, is also an important method by which the level of chronic virus excretors is maintained.

There is no evidence that pestiviruses can establish persistent infections in animals exposed to virus at times other than early pregnancy. BVDV could be detected for up to 103 days in susceptible calves infected at birth, and although 2 calves did not produce detectable levels of virus neutralising antibodies for 42 and 73 days the majority had seroconverted by 21-28 days and all calves were immune to challenge (Lambert and Fernelius,1968).

The mechanisms involved in the establishment and maintenance of persistent virus infections are complex, but low or no cytopathology and the ability to infect lymphocytes and macrophages are important properties (Mims,1974). The lack of cytopathogenicity has been confirmed for BVDV and BDV. BVDV has been shown to replicate in both cell types (Truitt and Shechmeister,1973) and a strong association of viral antigen with cells of the mononuclear phagocyte system has been demonstrated in inoculated foetuses (Ohmann<sup>et al.</sup>1982). Some control of viral replication must be exerted since some persistently infected immunotolerant animals can survive for years as clinically normal animals. An unstable equilibrium appears to be established between the virus and host.

ENTERIC DISEASE OF CATTLEBVD and MD

Two syndromes were described originally. BVD was very contagious with moderate clinical disease and pathological changes and had a high morbidity and low mortality (Olafson et al., 1946). This syndrome most probably resulted from the introduction of a virulent strain of virus into a naive herd. Such an epidemic form of infection is now rarely encountered. More commonly seen is the sporadic form of infection. This was originally named MD and had a low morbidity, was not highly contagious but affected animals had severe clinical signs and pathological lesions and the mortality rate was high (Ramsey and Chivers, 1953). These two names have been sustained because for some outbreaks they remain clinically and pathologically valid. Not all outbreaks conform to the high morbidity/low mortality or high mortality/low morbidity patterns. The two syndromes represent overlapping extremes at either end of a range of disease.

Experimental transmission studies, using filtered inocula from clinical cases, confirmed that following infection by several routes virtually all susceptible calves suffered only a mild disease. Pyrexia and leucopaenia were the only constant features. Oral hyperaemia with or without erosions, anorexia, depression, dyspnoea, serous nasal discharge and transient diarrhoea were other regular findings. The same disease was reproduced whether the inocula were from cases designated BVD or from animals dying of severe MD (Baker et al., 1954;

FIGURE 1.5      A confirmed case of clinical mucosal disease. Principal symptoms were depression and diarrhoea (Top), and crusting over muzzle and around eyes, resulting from excess nasal and ocular discharges (Bottom).



Huck,1957; Jarrett,1958; Pritchard,1963; Thomson and Savan,1963; Taylor et al.,1963; Tyler and Ramsey,1965). Occasional animals suffered severe ulceration lasting several weeks (Jarrett,1958), and 5-10% developed chronic lesions following acute infections (Pritchard,1963). In all the early transmission studies there were only 2 reports of deaths occurring. Baker et al. (1954) inoculated 100 calves of which 85 were susceptible to the virus. Only two animals died 11 and 14 days after inoculation and interestingly these both had hyperkeratosis, a possible indication of chronic persistent BVDV infection, at the time of inoculation. At post-mortem examination they showed ulcers in the mouth and extensive pneumonia, features that were not common in 19 other calves killed 4-16 days after inoculation. Pritchard (1963) recorded deaths in two calves 3 and 42 days after inoculation without providing details.

Inability to reproduce fatal mucosal disease has been for many years an enigmatic feature of BVDV infections. In addressing this problem Malmquist (1968) reported the detection of 4 unthrifty calves that had persistent viraemia and failed to produce neutralising antibodies. He attributed this failure to respond to the virus to immunological tolerance which could be acquired by intrauterine infections. It has been well established that animals succumbing to both acute and chronic MD are viraemic and have no, or very low, levels of neutralising antibodies to BVDV (Borgen,1963; Thomson and Savan,1963; Schaal et al.,1972). These features are common to

persistently infected calves. Fatal cases of MD amongst congenitally infected calves have been attributed to immunopathological mechanisms (Liess et al.,1974). In another important study, it was shown that animals dying of MD could be immunologically tolerant to one particular strain of BVDV but immunocompetent to another strain (Steck et al.,1980). The pathogenesis of MD remains largely unresolved.

#### Neonatal Enteric Disease

Both in-utero and post-natal infection with BVDV can result in enteric disease in the neonatal period. Severe in-utero infection caused 30 stillbirths and the death of 27 calves in one herd of 286 cows. Oral and lip lesions were seen at birth in both stillborn and live calves. Affected calves developed diarrhoea soon after birth and died between 2 and 13 days old (Romvary,1965a).

Post-natal infection with BVDV has been studied in specific-pathogen free calves. Eighteen calves were exposed orally or intranasally on the day of birth or at 3 days old. All developed mild to severe diarrhoea which persisted for 7 to 10 days and 4 of 13 colostrum-deprived calves died (Lambert et al. ,1974).

#### RESPIRATORY DISEASE IN CATTLE

In spite of the apparent association of BVDV with respiratory disease in field outbreaks, experimental infections have resulted in only mild lesions. Exposure of calves to BVDV aerosols produced only mild interstitial pneumonitis in two separate studies (Mills,1966;



Phillip,1973). In a third study no lung lesions were produced nor was there impaired clearance of Pasteurella haemolytica (Lopez et al.,1982).

#### DIAGNOSIS

Laboratory confirmation is required for the unequivocal diagnosis of the diseases caused by BVDV and BDV. As has been noted, congenital infection can result in the delivery of young infected with virus or with serum neutralising antibody. Tests for both must, therefore, be undertaken to establish whether or not in-utero infection has occurred.

Virus can be readily isolated from leukocytes of persistently infected cattle using bovine tissue culture systems (Malmquist,1968). Tests for NCP viruses are essential, and two methods have been used to detect BVDV and BDV in tissue culture. Interference tests (Gillespie et al.,1962; Hadjisavvas et al.,1975), and immunofluorescent tests (Fernelius and Lambert,1969; Harkness et al.,1977). Immunofluorescence and immunoperoxidase methods have also been used to demonstrate antigen in cryostat sections of organs from persistently infected animals (Terpstra,1978; Gardiner,1980; Ohmann et al, 1981). Comparative studies of direct detection of BVDV in organ sections and virus isolation in tissue culture with additional immunofluorescence staining for NCP virus showed that the latter method was three times more sensitive than the former for confirming BVDV infection in dead cattle (Bechmann et al.,1977).

The transitory nature of many postnatal infections makes virus isolation difficult, so that retrospective serology on acute and convalescent sera from several animals may be the only way of confirming recent infection.

#### CONTROL

The control of BVDV infections in some countries has been based on the use of modified live virus vaccines. The use of such vaccines has been associated with the production of disease. In a thorough investigation involving 9,891 cattle in 17 herds, Peter et al. (1967) reported that the disease which occurred was similar to the field form of MD. The average morbidity rate was 5.2% and the mortality rate among affected animals was estimated at 75%. Dying calves failed to seroconvert to BVDV but seroconversion occurred in calves recovering after having signs of MD and in those with no evidence of disease. The authors concluded that the condition was primarily caused by failure of the immune mechanisms in a few animals. Other studies have also shown that the use of live vaccines can be detrimental to some animals (Steck et al., 1980). Loss of confidence with live vaccines has led to the development of inactivated ones but these have not been widely evaluated in the field (Fernelius et al., 1972; McClurkin & Coria, 1978).

The use of live and inactivated vaccines containing BDV has been investigated experimentally. Double vaccination of ewes with certain inactivated virus preparations produced good antibody titres which protected

more than 50% of fetuses after the ewes had been challenged (Vantsis et al.,1980)

#### EPIDEMIOLOGY

The natural host range of Pestiviruses is restricted to ruminants and pigs. Among domestic animals experimental cross-infection has been demonstrated between cattle, sheep,pigs and goats (Ward,1971; Phillip and Darbyshire, 1972; French et al.,1974; Gibbons et al.,1974; Taylor et al.,1977). Interspecies infection, by some pestivirus strains at least, is therefore a possibility. There is serological evidence of widespread infections in a variety of wild ruminants, which should also be considered potential reservoir hosts. Positive results from African species with no known contact with domestic livestock suggest a longstanding natural infection (Hamblin and Hedger,1979; Karstad,1981).

British farming methods are not conducive to the exchange of pestiviruses from pigs to other susceptible species. Cattle and sheep, however, are often farmed together and shared grazing systems are encouraged for parasite control. Experimental cross infectivity is well documented but only circumstantial evidence of natural infection from cattle to sheep has been described (Physick-Sheard et al.,1980). Serological studies of cattle and sheep populations in several countries have confirmed that cattle are the principal hosts of pestiviruses. Thirty to 80% of adult cattle have antibody

to BVDV, the equivalent figure for sheep being 5 to 11% (Harkness and Vantsis, 1982). This difference in frequency of infection between cattle and sheep could be explained by variable opportunities for spread of infection among and between species arising from different types of management. Alternatively, cattle may be more susceptible to infection with pestiviruses or some strains may only be able to replicate in cattle.

#### AIMS OF THIS THESIS

The work reported in this thesis had two aims, both relevant to future pestivirus control policies.

The first was to investigate the response of normal lambs and those persistently infected with BD virus to further infection with pestiviruses. It was hoped that this would provide some insight into the outcome of the use of modified live virus vaccines in these two classes of animals.

The second aim was to study the degree of antigenic relatedness between reference and field strains of BVDV and BDV.

Initially, however, investigations were undertaken to establish a common tissue culture system for the propagation and assay of these viruses.

## CHAPTER 2

### GENERAL MATERIALS AND METHODS

#### INTRODUCTION

The contents of this chapter describe the basic technical work. More specialised procedures are included in the appropriate sections.

#### TISSUE CULTURE TECHNIQUES

Medium. The growth medium used for nearly all cell cultures was Eagle's minimal essential medium (MEM) (Eagle, 1959) supplemented with lactalbumin hydrolysate (LAH) (0.5%), glucose (0.2%) and 10% heat inactivated calf serum (56°C for 45 minutes), and containing 100 units per ml penicillin, 100 µg per ml streptomycin and 2 µg per ml amphotericin B deoxycholate. In some experiments Medium '199' (Morgan et al., 1950) replaced the MEM.

The maintenance medium for all cells was '199' medium supplemented with 0.5% bovine serum albumin (BSA), 0.1% lactalbumin hydrolysate (LAH) and 0.1% yeast extract, and containing the same antibiotics as in the growth medium. The heat-inactivated serum supplement for virus isolations was foetal calf serum (2%) and for virus growth it was 3% horse serum.

Cell culture procedures. Bovine and ovine fetuses contained within unopened uteri were collected from local abattoirs and transported to the laboratory. Primary and secondary cultures of foetal tissues were prepared using the trypsin disaggregation method described by Paul (1970a) for monkey kidney cell cultures, except that the growth medium for the pooled cell suspensions was supplemented Eagle's MEM as described above.

Semi-continuous and established cell lines were cultured using standard techniques (Paul,1970b).

#### VIROLOGICAL TECHNIQUES.

Viruses. Two cytopathic reference strains of virus were used throughout this work. The BVD virus was originally isolated from the spleen of a naturally occurring fatal case of BVD in the closed herd at National Animal Disease Laboratory, Iowa, U.S.A., and is referred to as BVDV-NADL (Gutekunst and Malmquist,1963). The BD virus was isolated at Moredun from a pool of brains from newborn lambs clinically affected with BD, and is referred to as BDV-M (Vantsis et al.,1976).

All other viruses, details of which are shown in Table 2.1, were isolated from clinical cases of BVD or BD in Scotland at the virus diagnostic laboratory, Moredun Research Institute.

#### Preparation of virus stocks.

Reference strains. Stocks of BVDV-NADL at an unknown passage level, and BDV-M at the 28th passage level were kindly supplied by Dr. D.R. Snodgrass, Microbiology Department, Moredun Research Institute.

BVDV-NADL, derived from the American type culture collection (ATCC VR-534), which had been passaged 7 times in bovine cells was kindly supplied by Dr. P. Roeder, Central Veterinary Laboratory, Weybridge; this virus was amplified by two further passages in bovine cells at low multiplicity of infection (m.o.i.) and stored as a master stock in small aliquots at -70°C.



A similar master stock of BDV-M was established at the 3rd FLK passage level by culturing a homogenate of the BD IIB brain pool (Barlow,1972) kindly supplied by Mr. A.C. Gardiner, Moredun Research Institute.

For all pathogenicity studies virus inocula were prepared from the lowest passage level available. For antigenic studies the viruses were passaged further including three plaque purifications in cultures of foetal lamb brain (FLB) cells. To minimise the production of defective interfering particles a low m.o.i. was used to produce new virus stocks.

Other isolates. Small stocks of all the other isolates were available at the second passage level in BEK (bovine isolates) or FLK cells (ovine isolates). All the viruses were purified by three plaque purifications (CP isolates) or three passes at terminal dilution (NCP isolates) in FLB cells. Master stocks of the viruses were prepared as for the reference strains and working stocks grown from these as required.

Titration of virus infectivity. Serial ten-fold dilutions of the virus suspension were made in maintenance medium in bijou bottles.

Initially, titrations were done in test tube cell cultures. Unless stated otherwise bovine isolates were titrated in secondary BEK cultures and ovine isolates in secondary FLK cultures. Cells were grown on the wall of 15 cm x 1.5 cm glass test tubes or on 6 x 22 mm coverslips inside tubes. Cultures at the point of confluence were washed three times with Hanks' basic salt solution (BSS).

An aliquot of each virus dilution was inoculated on to at least two tube cultures, and allowed to adsorb for 2 hours at 37°C. After this time 1 ml of maintenance medium was added to each tube, and cultures were reincubated at 37°C for up to 7 days. Those with characteristic cytopathic effect (CPE) 7 days after inoculation were scored as positive. When assaying for the presence of NCP virus coverslips were removed after 5 days' incubation and examined for virus using an indirect immunofluorescence test (IIFT).

Titration of viruses in FLB cells were carried out using small volumes of reagents. Cytopathic viruses were titrated in 96 well plastic microplates, using 4 wells per dilution. Fifty  $\mu$ l of virus were added to each well followed by 100  $\mu$ l of 199 medium supplemented with 10% horse serum containing  $10^4$  cells. The plates were sealed, and incubated at 37°C for up to 7 days after which wells were examined for the presence of CPE. The titration of NCP viruses was carried out in Lab-Tek chamber/slides (Miles Scientific Ltd.). Fifty  $\mu$ l volumes were added to each of 4 wells per dilution followed by  $4 \times 10^4$  cells in 400  $\mu$ l of medium as used in microplates. After 5 days, cells were stained by an IIFT to detect virus.

Virus titres were expressed as the  $\log_{10}$  tissue culture infective dose 50 (TCID<sub>50</sub>) as calculated by the Karber method (Lennette and Schmidt, 1979).

Indirect immunofluorescence test to detect virus. All samples were coded to obscure their origin. After the removal of medium, coverslips or chamber/slides were washed

three times with prewarmed phosphate buffered saline (PBS), before being air-dried. The cells were then fixed in cold acetone for 10 minutes and re-dried. Predetermined optimal working dilutions of two anti-pestivirus sera were used in the first stage of the test. A hyperimmune serum against BVDV-NADL prepared in a gnotobiotic calf (Nuttall et al., 1977), kindly supplied by Dr. E.J. Stott, Institute for Research on Animal Diseases, Compton, Berkshire, was used for bovine isolates. A convalescent sheep serum against BDV-M (Gardiner, 1980) was used on ovine isolates. These sera were applied to the cells and incubated at 37°C for 30 minutes. Cells were washed in 3 changes of PBS for 20 minutes. An optimal dilution of rabbit anti-sheep immunoglobulin conjugated to fluorescein isothiocyanate (Wellcome Reagents Ltd., Beckenham, England) was applied to all cells as the second stage serum. After incubation at 37°C for 30 minutes cells were washed as before, air-dried, and mounted under phosphate buffered glycerol (pH 8.2). Known infected and uninfected cells were included in every test.

For the examination of cells for specific fluorescence a Leitz Ortholux 2 microscope was used. This was fitted with a pleomopak 2 vertical illuminator employing an incident light source from a 50 Watt ultra-high pressure mercury lamp.

Virus isolation. Swabs were collected into separate bottles of 4 ml Hanks' BSS containing 1% BSA, 300 iu/ml penicillin, 300 µg/ml streptomycin and 50 iu/ml polymixin B (virus transport medium (VTM)). Tissues were collected

aseptically into the same volume of VTM to give an approximate 1 to 10 ratio of tissue to fluid. Swabs were sonicated for 30 seconds in an ultrasonic waterbath (Engisonic Ltd., model B32), and the medium passed through 0.45  $\mu$ m filters. Tissues were homogenised, clarified by centrifugation at 2,000 x g for 10 minutes and the supernatant fluid passed through 0.45  $\mu$ m filters. Duplicate tubes of washed confluent monolayers of secondary FLK cells were each inoculated with 0.2 ml of test fluid. After two hours' adsorption at 37°C, 1 ml of maintenance medium was added to each tube.

To isolate virus from blood, samples were collected into 7 ml evacuated tubes (Vacutainers: Becton-Dickinson Ltd.), which contained 70 units of preservative-free heparin sodium BP (Evans Medical Ltd.). Tubes were centrifuged at 2,000 xg for 15 minutes at room temperature, the plasma was collected and the buffy coat was aspirated into 1 ml of maintenance medium. Cells were separated by vigorous pipetting before 0.2 ml of suspension was inoculated into each of 2 tubes of washed confluent monolayers of secondary FLK cells. After 2 hours' co-cultivation 2 washes with maintenance medium were used to remove the inoculum, and 1 ml of maintenance medium was added to each tube.

Inoculated tubes were incubated at 37°C and examined regularly for virus CPE. After 7 days, cultures were frozen and thawed once and passaged onto further FLK monolayers. All specimens received a minimum of 2 such passages after which time frozen and thawed cultures were

passed onto FLK cells grown on coverslips. Seventy-two hours later cells were examined for the presence of pestivirus antigen by IIFT. Further passages onto cells in tubes were used to determine the cytopathogenicity of isolates.

Virus purity checks. Throughout this work the following tests were used to test the purity of virus stocks and isolates recovered from infected animals:-

Cytopathology of virus-infected cell cultures. At intervals after inoculation coverslip cultures were removed from tubes, washed in PBS, fixed in Bouin's solution, cleared in alcohol and stained with May-Grunwald stain followed by 10% Giemsa. Coverlips were differentiated in distilled water, blotted dry, rinsed in xylene and mounted in DPX.

Haemadsorption. At intervals after inoculation cells were washed three times in PBS and 0.2 ml of a 0.5 % suspension of guinea-pig erythrocytes was added to each tube. After 30 minutes incubation at 37 C, cells were washed thoroughly with PBS and then examined for the presence of haemadsorption. Uninoculated cells and cells infected with parainfluenza type 3 virus (Strain G2) (Hore, 1966) were used as controls.

Electron microscopy. Cells from cultures with evidence of virus-induced CPE were scraped from the glass and pelleted at 2000 x g for 10 minutes in conical-bottomed plastic universal bottles. Medium was drained off and the cells resuspended in a small volume of distilled water. The cell lysates were applied to a formvar/carbon coated grid and

negatively stained with 1% potassium phosphotungstic acid, pH 7.0 (Brenner and Horn, 1959).

Thin sections of infected and uninfected cells were also examined. Cells were fixed in freshly prepared 3% glutaraldehyde in phosphate buffer (pH 7.4), and post-fixed in 1% osmium tetroxide in phosphate buffer (pH 7.4). After dehydration in graded alcohols, cells were embedded in Araldite. Sections were cut on a Reichart OMU4 ultramicrotome and stained with uranyl acetate followed by lead citrate.

E.W. Gray and J.D. Menzies of the Electron Microscopy Section, Moredun Research Institute, carried out the electron microscope studies.

#### SEROLOGICAL TECHNIQUES

##### Serum-virus neutralisation tests.

All serum and plasma samples were heat-inactivated at 56°C for 30 minutes soon after collection and stored at -20°C until they were tested. To minimise the effect of test-to-test variations, samples collected at the one time from all animals in an experiment were included in the same test. The medium used for all dilutions and for cell growth was '199' supplemented with BSA (0.5%), yeast extract (0.1%) and 10% heat-inactivated horse serum. Antibiotics were included as detailed under tissue culture medium (see above).

Constant virus-varying serum. A microneutralisation test was used, based on the method of Rossi and Kiesel (1971).

Tests were carried out in 96-well microtitre plates. Serial two-fold dilution series (25 µl per well) of test



serum were prepared in duplicate, and an equal volume of virus containing approximately 100 TCID<sub>50</sub> was added to each well. Duplicate wells containing the lowest dilution of test serum without antigen were included as controls to detect cytotoxic sera. After incubation at 37°C for one hour, 100 µl of a suspension of FLB cells containing 10<sup>4</sup> cells were added to each well. Plates were sealed with non-toxic clear adhesive tape and incubated at 37°C. Cultures were examined for the presence of CPE after 5-7 days and the result expressed as the reciprocal of the serum dilution corresponding to the 50 per cent end-point of neutralisation as calculated by the Karber method (Lennette and Schmidt, 1979). The actual titre of virus used in the test was always determined and if this was outside the range of 32-320 TCID<sub>50</sub> of virus per well the test was repeated. Known negative and positive sera were included in every test.

Constant serum-varying virus. Serial ten-fold dilutions of virus were prepared in bijoux bottles. A 0.2 ml aliquot of each dilution of virus was added to an equal volume of a 1 in 20 dilution of test or control antiserum. After incubation at 37°C in a water bath for 1 hour residual infectious virus was titrated in FLB cells as described above. The serum neutralisation index was calculated by subtracting the log<sub>10</sub> titre of virus incubated with the test serum from the log<sub>10</sub> titre of virus incubated with the control serum.

Immunofluorescence test for titrating antibodies in sera.

Virus was diluted so as to contain sufficient infectious

particles to infect approximately 50% of cells in confluent FLB cells grown on coverslips in tubes. Seventy-two hours after infection coverslips were washed, fixed in cold acetone and either used immediately or stored at  $-20^{\circ}\text{C}$  in boxes containing silica gel.

Serial two-fold serum dilutions in PBS were made in microplates and duplicate coverslips were each overlaid with 25  $\mu\text{l}$  of dilutions of test sera. After incubation at  $37^{\circ}\text{C}$  for 30 minutes cells were washed in three changes of PBS for 20 minutes. An optimal working dilution of rabbit anti-sheep immunoglobulin conjugated to fluorescein isothiocyanate (Wellcome reagents Ltd, Beckenham, England) was used as the second stage serum as previously described. Cells were mounted under phosphate buffered glycerol (pH 8.2) and examined for evidence of characteristic immunofluorescence. Low dilutions of sera were also tested on uninfected cells. Before reading, the origin of each coverslip was obscured. Coverslips were recorded as being positive or negative for immunofluorescence and the result expressed as the reciprocal of the serum dilution corresponding to the 50% end-point of detectable fluorescence as calculated by the Karber method (Lennette and Schmidt, 1979).

#### HAEMATOLOGY

All blood samples were collected into evacuated glass tubes containing 10 units of preservative-free heparin sodium BP per ml of collected blood.

Haematological examinations from experimental animals were carried out according to the methods described in Schalm et al. (1975). Blood cell counts were made using an electronic particle counter (Coulter Counter; model ZBl). Haemoglobin was measured in a haemoglobinometer (Coulter Electronics Ltd., Harpenden, Herts.), and Packed cell volume (PCV) was determined using a micro-haematocrit (MSE Ltd., Crawley, West Sussex).

Blood smears for differential leukocyte counts were fixed in methanol and stained with 10% Giemsa stain for 30 minutes. Smears were differentiated in distilled water and air-dried. Two hundred leukocytes were identified and the results expressed as percentage of total count and as absolute numbers per  $\mu$ l of blood.

#### PATHOLOGY

Animals either died or were exsanguinated under deep sodium pentobarbitone anaesthesia. At necropsy, the entire central nervous system (CNS) and representative samples of other organs and tissues were fixed by immersion in Baker's calcium formol; blocks were taken, processed to paraffin, sectioned and examined by light microscopy. Stains used included haematoxylin and eosin (HE), luxol fast blue, alcian blue-periodic acid schiff, methyl green pyronin and Feulgen.

Dr. R.M. Barlow of the Pathology Department, Moredun Research Institute, carried out the pathological studies.

### BACTERIOLOGY

Cell cultures, virus stocks and animal tissues were tested for the presence of bacteria by inoculating broth cultures and by streaking out on 7% sheep blood agar plates. Following incubation at 37°C for 24 or 48 hours bacteria were identified on the basis of their colony morphology on blood agar and by Gram stain. Broth cultures were sub-cultured on to blood agar if direct cultures onto blood agar yielded few or no colonies.

Mr. J. Fraser of the Bacteriology Section, Moredun Research Institute, carried out the bacteriological examinations.

### MYCOPLASMOLOGY

Cell cultures, virus stocks and animal tissues were tested for mycoplasma contamination by inoculating 0.2 ml of a 1 in 10 suspension of sample into 2 ml each of OB, UB and AB mycoplasma broth medium and by streaking out a loopful of suspension on OA agar (Jones, 1978). Isolates were identified by growth inhibition tests or indirect immunofluorescence tests.

Mr. A.G. Rae of the Mycoplasma Section of the Microbiology Department, Moredun Institute, carried out the examinations for mycoplasma.

TABLE 2.1

Details of field isolates of BVD and BD viruses

Designation	Date and place of origin	Animal	Disease
CP BVDV-7446	11.9.78 Grampian	18 month old bullock	Acute MD
CP BVDV-8085	30.1.79 Highland	6 month old heifer	Acute MD
CP BVDV-B816	20.4.81 Borders	6 month old heifer	Acute MD
NCP BVDV-D1787	28.7.82 Grampian	Bovine foetus	Abortion by heifer
NCP BVDV-E3679	3.11.83 Borders	5 month old heifer	Chronic MD
NCP BVDV-G982	5.4.84 Dumfries and Galloway	17 month old bull	Sight defect (Allison, 1984)
NCP BDV-B1056	20.5.81 Lothian/ Moredun	3 day old lamb	BD lamb born to ewe injected with H77 virus ex field disease (Vantsis et al., 1980 )
NCP BDV-G130	30.4.84 Grampian	Young lamb	"Hairy- shaker"
NCP BDV-G2048	14.7.84 Strathclyde	3 month old lamb	Survivor of BD abortion outbreak. No classical BD lambs born.

MD - Mucosal disease      CP - Cytopathic isolate

NCP - Non-cytopathic isolate

BVDV - Bovine virus diarrhoea virus

BDV - Border disease virus

CHAPTER 3CELL CULTURE STUDIES WITH BVD AND BD VIRUSESINTRODUCTION

Reliable cell culture methods were required for the propagation and assay of BVD and BD viruses. The susceptibility of a range of cell cultures to BVDV has been documented and the virus has been shown to replicate in a number of bovine cell lines as well as in low passage bovine cells (Saurat et al., 1972). Studies with BDV, however, have usually used low passage ovine or bovine cells for virus growth.

Continuous cell lines have two outstanding advantages that make them attractive for this kind of study. Their ready availability allows large stocks to be produced so that all experiments can be conducted using few passages from the starting material. Stocks can be thoroughly screened for the presence of 'wild' viruses: this is particularly useful since BVDV is a major contaminant of bovine foetal cells (Kniazeff, 1973). One cell line, derived from foetal lamb muscle, has been used for the growth and assay of BVDV and BDV (Laude and Gelfi, 1979). Unfortunately, this cell line is no longer available (Laude, personal communication).

In this laboratory previous studies have utilised secondary BEK cells for the assay of BVDV-NADL, and secondary FLK cells for the assay of BDV-M. Comparative serum neutralisation tests using these systems have consistently detected differences in the humoral antibody



response of sheep to infection with various BVD and BD viruses (Vantsis et al., 1976; ~~Vantsis et al., 1980~~ ; Vantsis et al., 1980 ). It has, been recognised, however, that the use of the two assay systems has meant that comparison of the titres to each virus ought to be made with caution (Vantsis et al., 1979). A single cell system for the assay of the two viruses would be advantageous since the amount of neutralisation in serum-virus interactions is influenced considerably by the host cell system in which residual virus infectivity is assayed (Della-Porta and Westaway, 1977).

This chapter is concerned with several aspects of the in-vitro cultivation of BVDV and BDV which were investigated in attempts to establish a single reliable cell culture system for the growth and assay of both viruses.

#### SECTION 1. THE SUSCEPTIBILITY OF DIFFERENT CELL CULTURES TO BVD AND BD VIRUSES.

Established cell lines, low-passage (semi-continuous) cell lines established from foetal organs, and secondary cells were tested for their susceptibility to BVD and BD viruses.

#### MATERIALS AND METHODS

Viruses. BVDV-NADL had been passaged an unknown number of times in bovine kidney cells. BDV-M had been passaged 28 times in FLK cells. Working stocks of these viruses were stored as 1 ml aliquots at -70°C and used as required.

Cell cultures and infectivity assays. The cell cultures tested and their passage histories are shown in the Tables

in the Results section. Cells were grown to confluence on coverslips in glass test tubes, and infected with serial ten-fold dilutions of virus as described in Chapter 2.

The cells were examined for evidence of virus-induced CPE at 5, 8 and 10 days. If no CPE was detected in cells infected with BD virus, coverslips were removed from tubes receiving low dilutions of virus, and the cells were stained by IFT to detect any evidence of replicating virus. Uninfected control cells were also examined for the presence of virus by IFT.

Statistical Analysis. The infectivity titres of the two viruses in secondary BEK and FLK cultures were compared using 2x2 factorial analysis of variance. The level of significance of differences for comparisons with significant F-ratios was established using an honestly significant (HSD) test (Runyon and Haber, 1980).

## RESULTS

Titration of stock viruses in secondary BEK and FLK cultures. The results of five replicate tests, read 10 days after inoculation, are shown in Table 3.1. The mean infectivity titre of BVDV-NADL was higher in BEK than in FLK cells, but the difference was not significant. The mean infectivity titre of BDV-M, however, was significantly higher ( $p < 0.01$ ) in FLK cells than in BEK cells.

Established cell lines. The results are summarised in Table 3.2 from which it can be seen that both viruses failed to cause CPE in any of the lines tested. The IFT failed to show any evidence of BD virus replication in cell

TABLE 3.1

Titration of stock viruses in secondary BEK and FLK cells

BEK CELLS			FLK CELLS		
Batch No.	BVDV-NADL	BDV-M	Batch No.	BVDV-NADL	BDV-M
272	5.0 *	2.0	273	4.5	4.5
278	5.5	3.5	277	4.5	4.0
297	4.5	2.5	306	5.5	4.5
308	5.5	2.5	313	4.5	4.5
312	4.0	3.5	315	3.5	3.5
Mean	<u>4.9</u>	<u>2.8</u>		<u>4.5</u>	<u>4.2</u>

\*  $\text{Log}_{10} \text{TCID}_{50}$  per 0.2 ml virus

TABLE 3.2

Titration of stock viruses in established cell lines

Cell Line	Passage Number	BVDV-NADL CPE after 10 days	BDV-M CPE after 10 days	IFT
<hr/>				
Mouse Cells				
L929	622	---	---	-
McCoy	?	---	---	-
Hamster Cells				
BHK-21	152	---	---	-
Monkey Cells				
LLCMK-2	306	---	---	-
Vero	236	---	---	-
Rabbit Cells				
SIRC	493	---	---	+
RK-13	123	---	---	+C+
Pig Cells				
PK-Pirbright	85	---	---	+C+
Bovine Cells				
MDBK	146	---	---	+C+

? = Not Known      --- = No CPE at a virus dilution of  $10^{-1}$

- = No fluorescence at a virus dilution of  $10^{-1}$       + = Fluorescence detected at a virus dilution of  $10^{-1}$

+C+ = Fluorescence detected in uninfected control cells as well as infected cells

lines derived from mouse, monkey or hamster tissues. Of the two rabbit cell lines tested, specific fluorescence was seen in both. With 1/10 dilutions of virus, fluorescence was restricted to two small areas of SIRC cells on one of the coverslips; no fluorescence was seen in cells infected with higher dilutions of virus or in uninfected SIRC cells. In RK-13 cells fluorescence was apparent in many cells infected with low dilutions of virus; uninfected RK-13 cells showed a similar pattern of widespread fluorescence. Similarly, the PK-Pirbright and MDBK cell lines showed virtually all cells to be fluorescing whether they were infected or not. No fluorescence was detected when the first stage serum was replaced with a calf serum without detectable neutralising antibody to BVDV-NADL or BDV-M, and it was concluded that the RK-13, PK-Pirbright and MDBK cell lines were persistently infected with a BD-related virus.

Semi-continuous cell lines and secondary cells. The results are summarised in Table 3.3. Several clear results emerged from these titrations. Cells of ovine origin were, with only one exception, susceptible to both viruses. Their sensitivity for the assay of BD-M was shown by the fact that the final titre was apparent by the fifth day. In contrast to this, slower development of the CPE in cells infected with BVDV-NADL meant that the final titre was not reached until the eighth day. Comparable titres of BDV-M were obtained in all the cells tested with the exception of the foetal lamb (FL) lung cells. Sheep thyroid, FL skin and FL brain cells were the most sensitive for the assay of BVDV-NADL and it was encouraging that even after 38 passages the FL brain cells were sensitive to both viruses.

TABLE 3.3

Titration of stock BVD and BD viruses in semi-continuous cell lines and secondary cells of ovine, bovine and porcine origin

Cell Type	Passage Number	BVDV-NADL			BDV-M		
		5#	8	10	5	8	10
<hr/>							
<u>OVINE</u>							
Sheep Thyroid	3	3.2*	4.5	4.5	4.2	4.2	4.2
Foetal Lamb (FL) Muscle	2	3.0	3.5	3.5	4.0	4.0	4.0
FL Spleen	13	1.5	2.5	2.5	4.5	4.5	4.5
FL Skin	15	3.0	4.5	4.5	4.5	5.0	5.0
FL Brain	8	4.0	4.0	4.5	3.5	3.5	4.0
FL Lung	12	3.0	3.0	3.0	<1.0	<1.0	<1.0
<u>BOVINE</u>							
Bovine embryonic (BE) Trachea	2	3.5	3.5	3.5	<1.0	<1.0	<1.0
BE Turbinate	0	4.5	4.5	4.5	1.5	2.5	2.5
BE Skin	2	5.0	5.0	5.0	<1.0	<1.0	<1.0
BE Testis	3	5.0	5.0	5.0	2.5	3.5	4.0
<u>PORCINE</u>							
Pig Kidney	2	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Pig Testis	3	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0

# Days post-inoculation when titres calculated

\* Results expressed as  $\text{Log}_{10} \text{TCID}_{50}$  per 0.2 ml virus



All the bovine cells were susceptible to BVD-NADL and only 5 days were required for the CPE to develop at terminal dilutions to give the final titres. The trachea cells at passage 32 were the least sensitive to BVD-NADL, but turbinate and testis cells at 20 and 13 passages respectively recorded high titres. These last two cell types showed some CPE when infected with BDV-M but this was slow to develop and only the testis cells gave a final titre comparable to those recorded in ovine cells.

No CPE developed in the two porcine cell types tested. Pig testis cells infected with both viruses stained by immunofluorescence were positive at titres of 2.5 and 3.5 for BVD-NADL and BD-M respectively.

#### CONCLUSIONS

None of the established cell lines tested were of value for this work. Whilst a range of cells of both bovine and ovine origin was susceptible to BVDV-NADL a more rapid CPE developed in the bovine cells. The BD virus was more fastidious in its cell requirement for growth. A rapid CPE at terminal dilution was seen only in ovine cells infected with BDV-M; replication with CPE did, however, occur slowly at low dilutions in some bovine cells. Both viruses showed evidence of replication in porcine cells but without the production of CPE.

#### SECTION 2. THE GROWTH OF BVD AND BD VIRUSES IN SELECTED CELL CULTURES FROM OVINE AND BOVINE FOETUSES

The results from Section 1 showed that cells derived from several foetal lamb organs were susceptible to both

viruses. The highest titres of the two viruses were recorded in foetal lamb kidney, skin and brain cells. Of the bovine embryonic tissues tested only testis cells showed good evidence of BD virus replication. In this section the ability of skin, testis and brain cultures made from ovine and bovine foetuses to produce infectious virus has been studied.

#### MATERIALS AND METHODS

Viruses. The high passage stocks of BVDV-NADL and BDV-M described in the previous section were used.

Cells. Primary skin, testis and brain cell cultures were established by trypsin disaggregation from each of three ovine foetuses of approximately the same age, and three bovine foetuses of approximately the same age. Cells were passaged as necessary, being used between the second and eighth pass.

Experimental Procedure. The method was based on that described by McFerran et al.(1972), in which test viruses were titrated over two passages in different cell culture systems followed by titration in kidney cells derived from the same species of animal as that from which the virus was originally isolated.

Newly confluent cell monolayers consisting of approximately  $2 \times 10^7$  cells grown in Sani/Glass (S/G) bottles were washed thoroughly with Hanks' BSS and infected with 1 ml of stock virus. Fifty ml of maintenance medium were added to each bottle and the cells incubated at 37°C for 5 days. After this time the state of the cell monolayer was recorded and the supernatant fluid collected. After

clarification at 2000xg for 15 minutes aliquots of the fluid were stored at -70°C. For the second passage onto cells in S/G bottles 10 ml of first harvest supernatant were used as inoculum, and removed before maintenance medium was added. Cells were incubated for 5 days, examined and virus recovered and stored as before. Virus yields from both passes were titrated in tube cultures of homologous cells, and yields from the second passage were also titrated in FLK and BEK cells. All titrations were read 5 days after inoculation.

Statistical analysis of results. Virus infectivity titres in different cells at the two passage levels were compared using 2 x 6 or 3 x 6 factorial analysis of variance. The level of significance of differences for comparisons with significant F - ratios was established using an HSD test.

## RESULTS

Cell Growth. All cells were grown successfully with one exception. The brain culture from the first lamb stopped growing after 5 passages before the second virus passage had been undertaken. No cause was established. A brain culture from a fourth lamb was used for the second virus passage and virus titrations.

Development of CPE in cells grown in S/G bottles. In comparison to uninfected control cells a clear CPE was seen in all cell types infected with BVDV-NADL at both passages. More than 50% of cells in the monolayers were showing evidence of degeneration and many had become detached from the glass. Some evidence of CPE was apparent in all the ovine cell types infected with BDV-M but this was generally

far less advanced than that seen in BVD virus-infected cells. Apart from the degree, there was no discernible difference between the effects produced by the two viruses. In bovine cells, BDV-M-induced CPE was not extensive but was apparent in all but the skin culture of one calf at the first pass, and the skin culture of the other two calves at the second pass. One brain culture at the first pass was also free of detectable CPE.

Virus yields from different cell types. The results of the virus titrations in homologous cells after passage are shown in Table 3.4. All titres were lower than those previously obtained with the stock viruses in these cell types. After 1 passage, there were no significant differences among the virus yields from the three ovine cell types. Combined virus yields from BE skin and BE brain cells, but not BE testis cells, were significantly lower ( $p < 0.01$ ) than yields from the ovine cells. Since there was no significant difference between yields of BVD-NADL from any of the cell types from either species, disparity between combined virus yields from ovine and bovine cells was due to the reduced growth of BDV-M in bovine cells. Comparison of the yields of BDV-M from cells of the two species showed that the reduced growth was significant in all cell types, brain ( $p < 0.001$ ), skin ( $p < 0.01$ ) and testis ( $p < 0.05$ ).

After 2 passages, the reduction in growth of BDV-M in bovine cells was even more marked, brain ( $p < 0.001$ ), skin ( $p < 0.001$ ) and testis ( $p < 0.01$ ). One other significant finding was the lower yield of BVDV-NADL from BES cells compared to FLS cells ( $p < 0.05$ ).

TABLE 3.4

Virus yields from cell cultures derived from ovine  
and bovine fetuses

Cell type	Pass 1		Pass 2	
	BVDV-NADL	BDV-M	BVDV-NADL	BDV-M
FL Skin	3.0 ± 1.8*	3.5 ± 0.5	3.5 ± 1.0	4.0 ± 1.3
FL Testis	3.3 ± 0.6	3.6 ± 1.0	3.6 ± 1.0	3.3 ± 0.3
FL Brain	2.5 ± 0.0	3.8 ± 0.8	3.2 ± 0.8	3.3 ± 0.3
BE Skin	2.5 ± 1.5	0.8 ± 0.8	2.0 ± 0.9	0.8 ± 0.8
BE Testis	3.2 ± 0.3	2.0 ± 0.5	3.2 ± 0.6	1.5 ± 1.0
BE Brain	2.3 ± 0.8	0.8 ± 0.8	3.2 ± 0.6	0.8 ± 0.3

\* Mean ± SD from 3 fetuses expressed as Log<sub>10</sub>

TCID<sub>50</sub> per 0.2ml of virus.

When the virus yields from the second passages in different cell types were assayed in secondary BEK and FLK cells some clear differences emerged (Table 3.5). The differences all involved the assay of BDV-M. Titration of BVDV-NADL in BEK cells gave slightly higher values than in the homologous cells. Overall, however, there were no significant variations in BVDV-NADL virus yields from any of the 6 cell types whether they were assayed in homologous cells, BEK cells or FLK cells.

The most striking of the results was that BDV-M had been produced to a high titre in the three bovine cell types but this had not been detected by assay in these cells. Only when FLK cells were used for titration was the relatively high concentration of virus detected. Analysis of BDV-M titres in FLK cells from the six cell types showed that virus yields from FLT and FLS cells were significantly lower ( $p < 0.05$ ) than yields from at least one of the bovine cell types; virus yields from FLB cells, however, were not significantly lower than yields from any of the bovine cells.

Comparison of BDV-M titres in homologous cells, BEK cells and FLK cells showed that FLK cells were significantly ( $p < 0.001$ ) the most sensitive for virus assay. Low virus titres in secondary BEK cells re-emphasised the unsuitability of bovine cells for the assay of BDV-M. Comparison of BDV-M virus titres in homologous and FLK cells showed that variation depended on the cells used for virus growth. Thus, virus grown in ovine cells gave



TABLE 3.5

Titration of stock viruses in secondary BEK and FLK cells after two passages in stated cell types compared with the titres obtained in those homologous cells

Cell Type	Titre in Homologous cells		Titre in BEK Cells		Titre in FLK Cells	
	BVDV-NADL	BDV-M	BVDV-NADL	BDV-M	BVDV-NADL	BDV-M
FL Skin	3.5* ±1.0	4.0 ±1.3	2.7 ±0.3	1.3 ±1.9	3.7 ±0.3	3.8 ±1.2
FL Testis	3.6 ±1.0	3.3 ±0.3	3.2 ±0.8	0.5 ±0.5	3.0 ±1.0	3.0 ±0.5
FL Brain	3.2 ±0.8	3.3 ±0.3	3.3 ±1.0	1.3 ±1.5	3.3 ±1.0	4.2 ±1.5
BE Skin	2.0 ±0.9	0.8 ±0.8	3.8 ±1.3	1.0 ±1.0	3.5 ±0.5	4.7 ±1.3
BE Testis	3.2 ±0.6	1.5 ±1.5	4.3 ±1.6	1.5 ±1.0	3.0 ±0.5	5.7 ±1.0
BE Brain	3.2 ±0.6	0.8 ±0.3	4.5 ±0.5	2.7 ±1.4	3.0 ±0.9	4.5 ±0.5

\* Mean ±SD from 3 foetuses expressed as Log<sub>10</sub> TCID<sub>50</sub> per 0.2ml of virus.

comparable titres in homologous and FLK cells, whereas virus grown in bovine cells gave significantly higher titres ( $p < 0.001$ ) when assayed in FLK cells.

#### CONCLUSIONS

There were no significant differences between the yields of BVDV-NADL from any of the six cell types used. After two passages in these cell types virus yields were comparable whether assayed in homologous cells, BEK cells or FLK cells.

The apparent yields of BDV-M from the six cell types varied according to which cells were used to assay the virus. The most sensitive cells for assaying BDV-M were FLK cells. When these were used for virus assay, it was apparent that the highest virus yield had been produced in bovine testis cells. This yield was not significantly different from the yields from the other bovine cell types and the FL brain cells.

The best growth system for the two viruses appeared to be BE testis cells but bovine cells were consistently unsuitable for the assay of BD-M virus. All three ovine cell types, however, were sensitive indicators of the CPE produced by both viruses, but only FL brain cells had supported growth of BD-M to a level comparable with that obtained in the bovine cells. For this reason FL brain cells were used subsequently for comparative in-vitro studies on the antigenicity of BVD and BD viruses.

SECTION 3. INVESTIGATIONS INTO POSSIBLE SOURCES OF  
PESTIVIRUS CONTAMINATION OF SUSCEPTIBLE  
OVINE AND BOVINE CELLS

Having established the suitability of ovine cells of foetal origin for the cultivation and assay of both BVDV-NADL and BDV-M it was of interest to determine if latent pestivirus contamination of such cells was likely to pose a problem. Ovine cells have been used in studies on BVD viruses (Siebold and Dougherty, 1967; Singh, 1969a), but no reports of latent contamination of such cells are known. Pestivirus contamination of bovine cells, however, has been well documented and recognised as a serious disadvantage of using bovine tissue cultures. Contamination can be present in the cells themselves or can arise from the bovine serum supplement used in the growth medium (Nuttall et al., 1977). In some instances it was not possible to ascertain which of these two sources was the cause of the contamination (Rossi et al., 1980).

In this section pestivirus contamination rates of the sera most commonly used for supplementation of tissue culture medium, and bovine and ovine foetal cell cultures were studied.

SECTION 3.1. PESTIVIRUS CONTAMINATION OF BOVINE SERA.

MATERIALS AND METHODS.

Virus isolation. Six batches each of commercially available foetal bovine serum (FBS) and newborn calf serum (NBCS) were tested for the presence of pestiviruses. All the sera had been 'virus screened' by the suppliers. Thirty-five ml of each serum, not heat-inactivated, were

used as a 10% supplement of '199' medium for the growth of a semi-continuous cell line derived from foetal lamb brain known to be susceptible to BVD and BD virus. In each test, a batch of FBS and a batch of NBCS were used in parallel on the same cells. Fifty ml of test medium containing  $1 \times 10^5$  cells were added to a Sani-glass(SG) bottle. Cells were grown to confluence in 3-5 days, washed, trypsinised, resuspended in 150 ml of fresh growth medium, and seeded into 3 SG bottles. When confluent, cells from the 3 bottles were trypsinised, pooled, and resuspended in a further 150 ml of growth medium at a count of  $1 \times 10^5$  cells/ml. Confluent cells were trypsinised, pooled, resuspended as before and seeded into 6 test tubes containing coverslips. After four days, the coverslips were removed and the cells stained in an IIFT as described in Chapter 2. Three coverslips were tested with hyperimmune calf serum against BVD virus, and three with sheep anti-BD virus serum.

Measurement of neutralising antibody to BVD and BD viruses in commercial bovine sera. Heat-inactivated FBS and NBCS were tested for neutralising antibody to BVD and BD viruses in micro-neutralisation tests as described in Chapter 2.

RESULTS.

Pestivirus contamination of bovine sera. No virus was detected in FL brain cells grown in NBCS. Of the six batches of FBS tested, four were contaminated with non-cytopathic BVD virus. Specific fluorescence was widespread in cells on all coverslips stained with either of the two first stage sera.

Neutralising antibody to BVD and BD viruses in commercial bovine sera. The results are summarised in Table 3.6. All batches of NBCS had high levels of neutralising antibody to BVDV-NADL virus. The corresponding titres against BDV-M virus were always lower.

Of the 23 batches of FBS tested, fifteen had antibody to BVDV-NADL. This was generally detected at low serum dilutions only, but one batch had a reciprocal neutralising titre of 32. No neutralising antibody to BDV-M was present in any of the batches of FBS.

### SECTION 3.2. LATENT PESTIVIRUS CONTAMINATION OF BOVINE AND OVINE FOETUSES COLLECTED FROM ABATTOIRS.

In this section the results are presented of a three and a half year study on the latent pestivirus contamination rates of kidney cells grown from ovine and bovine foetuses collected from local abattoirs.

#### MATERIALS AND METHODS.

Source and culture of foetal material. Within five hours of slaughter, pregnant uteri were collected from local abattoirs and transported to the laboratory. Kidneys from both species were treated in the same way, being removed aseptically, chopped finely and disaggregated with 0.25% trypsin. The method was that described by Paul (1970a) for monkey kidney cells except that the growth medium for the pooled cell suspensions was Eagle's MEM supplemented with 10% bovine serum and containing 100 i.u/ml penicillin, 100 µg/ml streptomycin, 50 iu/ml polymixin-B and 2 µ/ml amphotericin B deoxycholate.

Cells were seeded into Thompson bottles and the

TABLE 3.6

Neutralising antibody to BVDV-NADL and BDV-M viruses in  
batches of bovine sera

	FBS		NBCS	
	BVDV-NADL	BDV-M	BVDV-NADL	BDV-M
No. of batches tested	23	23	12	12
No. of batches positive	15	0	12	12
Range of antibody titres	2-32	0	256-1024	6-128
Geometric mean titre	4	0	483	21



growth medium was changed to remove dead cells the following day. Confluent monolayers were produced within 3-5 days with foetal lamb kidney (FLK) cells showing faster growth than the bovine embryo kidney (BEK) cells. Confluent cells were trypsinised and  $10^5$  cells in 1 ml volumes were seeded into test tubes some of which contained coverslips. When monolayers became confluent they were washed three times with Hanks' BSS and then maintained with '199' medium supplemented with 0.5% BSA, 0.1% LAH, 0.1% yeast extract and 2% heat-inactivated foetal bovine serum free of anti-pestivirus antibodies; antibiotics were included in the same concentrations as used in the growth medium. Three days after the addition of maintenance medium duplicate coverslip cultures were examined for the presence of pestiviruses using an IIFT. Cells showing specific fluorescence were considered contaminated and all stocks of these cells were discarded.

Test tube cultures of IFT doubtful or negative cells were maintained for 1 week after which time cells were frozen and thawed once and 0.2 ml volumes of lysate were inoculated into further tubes of washed confluent homologous kidney cells free of pestivirus contamination. After 2 hours' adsorption maintenance medium was added and the cells incubated for a further week. During these blind passages cells were examined regularly for evidence of CPE. At the end of the second blind pass cells were frozen and thawed and 0.2 ml aliquots were added as before to at least 6 coverslip cultures of pestivirus-free BEK or FLK cells. Three days later the coverslip cultures were tested for the

presence of pestiviruses using an IFT. If there was no evidence of specific fluorescence the cells were considered to be free of contaminating pestiviruses.

Virus detection by IFT. This test was performed as described in Chapter 2. Two first stage sera were used. A hyperimmune serum against BVDV-NADL prepared in a gnotobiotic calf (Nuttall et al., 1977) was used on BEK cells and a convalescent sheep serum against BDV-M (Gardiner et al., 1980) was used on FLK cells. To test the ability of these two sera to detect noncytopathic pestiviruses from both cattle and sheep 12 BVD virus field isolates and 6 BD virus field isolates from disease outbreaks in Britain were used. These viruses had all been isolated at Moredun and stored at  $-70^{\circ}\text{C}$  as second pass stocks in BEK or FLK cells for up to 14 months. Volumes (0.2 ml) of bovine isolates were inoculated on to each of 4 washed monolayers of pestivirus-free BEK cells grown on coverslips.. After three days cells were stained in an indirect IFT, two coverslips receiving calf anti-BVD serum and two sheep anti-BD serum as the first stage sera. Sheep isolates inoculated onto pestivirus-free FLK cells were tested in the same way.

### Results

The 12 non-cytopathic BVD and 6 non-cytopathic BD virus isolates all showed clearly detectable specific immunofluorescence with both first stage sera.

The number of cell batches tested and the pestivirus contamination rates are summarised in Table 3.7. Of the 113 batches of BEK cells tested 15 were contaminated with

TABLE 3.7

Pestivirus contamination of foetal bovine (BEK) and  
foetal ovine (FLK) kidney cells

	BEK	FLK
Number cultured	113	105
Number contaminated	15	2
Percentage contamination	13.3	1.9

TABLE 3.8

Monthly Pestivirus contamination rates of BEK cells  
1980 - 1983

	<u>1980</u>	<u>1981</u>	<u>1982</u>	<u>1983</u>
January	-	1/5*	2/4	0/4
February	0/1	2/3	1/5	0/4
March	1/3	0/3	1/3	-
April	0/4	2/3	0/2	-
May	1/4	0/4	-	-
June	0/3	1/6	0/4	-
July	0/4	0/4	-	-
August	0/2	0/3	0/2	-
September	1/3	1/3	0/1	-
October	0/5	0/4	-	-
November	1/4	0/2	-	-
December	0/4	0/3	0/4	-
Totals	4/37	7/43	4/25	0/8

\* Number contaminated/Number tested

latent pestivirus infection. In twelve of these batches the contamination was detected in the first test on the secondary cells. Three other batches were detected as positive after further passage. The detection rate in FLK cells was significantly lower. Only two batches were contaminated and these were detected only after cell passage.

The monthly totals of the contamination rates of BEK cells are shown in Table 3.8. Although latent pestivirus infections were detected throughout the year, there was a tendency for this to be recognised in fetuses collected in the first four months of the year. Ten of 44 fetuses were positive during this time compared with 5 of 69 fetuses collected during the other 8 months. This difference was significant ( $\chi^2 = 4.3$ ;  $p < 0.05$ ).

The two contaminated batches of FLK cells were detected in fetuses collected in January 1981 and March 1982.

#### CONCLUSION.

Pestivirus contamination of FLK cells was detected, but the contamination rate was significantly lower than that of BEK cells.

#### DISCUSSION.

Titration of the cytopathic reference strains of BVD and BD viruses showed that the production of CPE was restricted to primary or semicontinuous cultures of ovine and bovine cells. Established cell lines of mouse, hamster and monkey tissue origin were refractory to both viruses;

no fluorescence was detected in cells infected with BDV-M or in uninfected cells. This finding compares with a previous report that BVDV-NADL and BVDV-C24V produced abortive infections, detectable only by immunofluorescence in hamster kidney and primate cells resembling the HeLa cell line (Fernelius et al., 1969b).

Of the two rabbit cell lines tested neither showed CPE but both had evidence of pestivirus replication as detected by immunofluorescence. In SIRC cells the small amount of fluorescence at a high virus concentration was due specifically to BDV-M but widespread fluorescence in both infected and uninfected RK-13 cells confirmed that this cell line was persistently infected with a virus related to BD-M. The susceptibility to pestiviruses of rabbit cells in-vitro has been reported previously (Hassan, 1979), and rabbits have been used for the serial passage of the non-cytopathic NY-I BVD virus and the NADL and C24V strains (Baker et al., 1954; Fernelius et al., 1969a). The bovine kidney and porcine kidney cell lines were also contaminated with a BDV-M related virus as judged by immunofluorescence, and were unsuitable for further study.

A wide range of tissues from ovine fetuses showed CPE with both viruses. Previous studies of BVD virus have utilised lamb testis cells (Siebold and Dougherty, 1967; Singh, 1969a). The second of these reports noted that the NADL strain caused a more slowly developing and less widespread CPE in the lamb cells than it did in BEK, BE lung and BE testis cells. It was also true of the findings

in this chapter that BVDV-NADL caused a more rapid CPE in the 4 bovine cell types studied. Nevertheless virus titrations showed that FLK, FL skin, FL brain and sheep thyroid cells were all equally susceptible to the two viruses.

Bovine foetal cells were not, in general, susceptible to BDV-M virus, as judged by the development of CPE. Only BE testis cells were likely to be of value even though the development of CPE was slow in these cells. Bovine testis cells have been used for studying cytopathic BD viruses (French et al.,1974) and non-cytopathic isolates ( Harkness et al.,1977; Terpstra,1981). One of the non-cytopathic isolates , Weybridge BD-2, was later shown to be cytopathic in a foetal lamb muscle cell line (Laude and Gelfi,1979).

The assay of the two viruses in porcine cells further demonstrated that the development of CPE was not always a reliable indicator of virus replication. Immunofluorescence tests were positive for both viruses and comparative titrations of them in pig testis cells suggested that this system was more sensitive to BDV-M than to BVDV-NADL.

Having established that both viruses were able to produce CPE in several ovine and bovine cell types, the ability of the three most promising cell types from the 2 species to produce infectious virus was tested. Previous work had shown that the ability of cells to detect a virus is correlated with their ability to produce infectious virus (McFerran et al.,1972). In these studies this was true of BVD-NADL virus in all 6 cell types, while for BDV-M



it was only true when the virus was grown and assayed in ovine cells. Growth of BDV-M in bovine cells produced a paradoxical result. When assayed in bovine cells virus growth appeared to be significantly less than that achieved in ovine cell types. Assay of the virus in FLK cells, however, showed that high levels of infectious virus had been produced by the bovine cells, particularly BE testis cells. This discrepancy was almost certainly exacerbated by the short period (5 days) allowed for CPE to develop, but it emphasised the unsuitability of bovine cells for the assay of BDV-M. In the wide range of viruses studied by McFerran and co-workers (1972) one other ovine virus, an adenovirus, gave a similar result.

Latent BVD virus contamination of foetal bovine serum and foetal bovine tissues has been widely recognised (Kniazeff, 1973; King and Harkness, 1975; Smithies and Modderman, 1975; Nuttall et al., 1977; Rossi et al., 1980).

In this laboratory the two major serum supplements of tissue culture growth medium are commercially supplied foetal bovine serum (FBS) and newborn calf serum (NBCS). Both sera are sold 'virus screened', which includes testing for BVD virus by a fluorescence test as well as checking for the presence of cytopathic agents. The suppliers' testing procedure would appear to be inadequate since NCP BVD virus was detected in 4 of the 6 batches of FBS tested. None of the 6 batches of NBCS contained BVD virus, and this was almost certainly due to the high levels of anti-pestivirus neutralising antibodies, which were found in all batches tested. It has been shown previously with

other bovine viruses that infectious virus could not be isolated from serum containing specific neutralising antibody even though electron microscopy revealed the presence of the viruses (Swack et al., 1975). Of the 23 batches of FBS tested, fifteen had neutralising activity against BVD-NADL, precluding their use in medium employed to maintain cells during virus growth.

Heat (56°C for 30 minutes) has been advocated for the inactivation of BVD virus in FBS. This has not always been effective, however (Nuttall et al., 1977; Rossi et al., 1980). There is also evidence that some isolates are more resistant to heat than others (Done et al., 1980). Susceptible cells grown in the presence of contaminated FBS may have to be subcultured several times before showing evidence of BVD virus infection (Nuttall<sup>et al.</sup>, 1977). Since it was envisaged that semi-continuous cell lines of ovine cells would be used for comparative studies of BVD and BD viruses it was felt to be essential that they were not exposed to potentially contaminated FBS.

Latent pestivirus contamination of foetal tissues results from transplacental infection. Isolation of virus from a variety of organs following experimental infection provides strong evidence of a viraemia in the foetus (French et al., 1974; Snowdon et al., 1975). The detection of latent BVD virus contamination in several bovine cell types would tend to support this view. In order to compare likely pestivirus contamination rates of locally available bovine and ovine fetuses, kidney cells were cultured from both species.

Pestivirus contamination was detected in 15 of 113 (13.3%) BEK cultures. Twelve of the cultures were positive at the second passage level, having been grown in medium supplemented with NBCS. Growth of cells latently infected with BVD virus in the presence of high levels of neutralising antibody has been reported previously and indicates that persistence is maintained by passage of the viral genome to each daughter cell during cell division (Coria and McClurkin, 1981). The BVD virus contamination rate found from this series of bovine embryos was comparable to the 20% detected in Belgium (Wellems and Leunen, 1974), the 10% detected in U.S.A. (Smithies and Modderman, 1975) and the 16% detected in an English survey (Nuttall et al., 1977). In each of these studies virus was detected by immunofluorescence. In a fourth study, a contamination rate of 40% was reported; of the 25 kidneys cultured, 7 were immunofluorescence positive, and 3 other kidneys were detected as positive by an interference test (Hassan, 1979). The interference test is not a sensitive test and variable results are obtained with different virus strains (Nuttall, 1977; Shirai et al., 1984). If the 3 kidneys detected as positive by the interference test alone are excluded the detection rate was 28%. One possible explanation for this high contamination rate could be that the fetuses were collected early in the year, since results reported in this Chapter showed that a significant number of positive fetuses was collected in the first four months of the year. Spread of virus among susceptible cows shortly after housing in the autumn might be one explanation of this finding.

Only two of 105 ovine kidney cultures had a latent pestivirus infection. This was another compelling reason for using ovine cells for studying BVD and BD viruses, but it demonstrated that regular screening of all cells would be needed. The prevalence of BD virus among Scottish sheep is low (Vantsis et al., 1979). In other areas of Britain, notably the English Midlands and Wales, serological surveys have demonstrated a higher prevalence (Sands and Harkness, 1978). The risk of pestivirus contamination of ovine fetuses collected in such areas could, therefore, be expected to be higher.

The work in this chapter was undertaken in an attempt to find a common cell system for the growth and assay of BVD and BD viruses. Low passage ovine foetal cells, particularly FL brain cells, were found to be suitable for the growth and assay of the two reference strains. The use of these cells for studying the antigenic relationships between field isolates and the two reference strains is described in Chapters 7 and 8.

CHAPTER 4THE EXPERIMENTAL PRODUCTION OF A MUCOSAL  
DISEASE-LIKE SYNDROME IN SHEEPINTRODUCTION

Lambs born with clinical symptoms of BD resulting from intrauterine exposure excrete virus for long periods of their lives (Gard et al.,1976; Plant et al.,1977). Such persistently infected lambs have been identified following experimental infection of ewes with tissue homogenates from BD lambs (Plant, Gard and Acland,1976), and BD and BVD viruses grown in cell cultures (Plant, Acland and Gard,1976; Sweasey et al.,1979; Terlecki et al.,1980; Terpstra,1981).

Persistent infections have also been detected in animals without clinical symptoms; some of these have shown symptoms as lambs and recovered, while others have been born apparently normal (Barlow et al.,1979; Westbury et al.,1979; Terpstra,1981).

Persistently infected sheep readily transmit infection to susceptible sheep, cattle and pigs (Barlow et al.,1980b; Terpstra, 1981). In addition, persistently infected lambs which survive and breed, frequently produce persistently infected offspring (Westbury et al.,1979; Barlow et al.,1980b; Gardiner and Barlow,1981). Such animals are, therefore, very potent sources of virus and their existence has a dominating influence on disease control strategies.

Virus persistence is possible only if the host's immune system fails to mount an effective attack on the

virus , or if the virus and virus-infected cells elude the attack. The mechanisms involved in the persistence of pestiviruses await elucidation. The usual finding that sheep persistently infected with pestiviruses have no anti-pestivirus neutralising antibodies has been interpreted as immunological tolerance (Gard et al.,1976). Persistently infected lambs can mount humoral antibody responses to other infectious agents, and occasional lambs produce anti-pestivirus neutralising antibody (Westbury et al.,1979; Terpstra,1981). These findings imply that if virus persists because of immunological tolerance, the tolerance is specific but may not be absolute.

In this chapter, two experiments are described in which normal lambs and lambs persistently infected with BD virus were challenged with BDV-M and BVDV-NADL viruses. The experiments were designed to test the stability and specificity of the putative immunological tolerance.

## MATERIALS AND METHODS

The preparation of cell cultures, virus stocks and the serological tests and virus isolation procedures used were as described in Chapter 2. High passage BDV-M and BVDV-NADL viruses were used for animal inoculation and serum neutralisation tests. The details of the actual inocula are given in the design of each experiment.

### EXPERIMENT 1

#### DESIGN OF EXPERIMENT.

Seven sheep without neutralising antibody to BDV-M or BVDV-NADL were used. Five were animals which had recovered



from clinical BD (recovered hairy shakers (RHS)), and 2 were from a flock in which there had never been any evidence of border disease. All the RHS had been born to ewes inoculated at 54 days gestation with the Moredun IIB brain pool (Barlow, 1972).

The seven sheep were each injected subcutaneously over the left shoulder with 2 ml of a clarified suspension of BDV-M grown in FLK cells from the 28th passage virus as described by Vantsis et al., (1980). The inoculum contained  $5.7 \log_{10} \text{TCID}_{50}$  per ml of infectious virus when assayed in tube cultures.

The humoral response of all animals was to be assessed by measurement of neutralising antibodies to BDV-M and BVDV-NADL at 3 weeks post-infection and at regular intervals thereafter.

## RESULTS

Clinical Observations. No clinical symptoms were seen in the two control sheep but, surprisingly, all 5 RHS became depressed 2 to 3 weeks after inoculation. Other symptoms also developed and are summarised in Table 4.1, together with the fate of the RHS. Sheep number 102A was the least affected of the group and recovered. It remained clinically normal until killed 273 days after inoculation.

Serology. Three weeks after injection the two control sheep both had SN titres of  $>320$  to BDV-M, while only 3 of the RHS showed any evidence of seroconversion with titres of 5 or 10. The final titres at time of death are shown in Table 4.1. Animal number 102A was seronegative at 3 weeks

TABLE 4.1

Experiment 1 - Clinical and serological response of RHS  
to infection with BDV-M virus

Animal	Sex/ Breed	Age (Mths)	Clinical Disease	Fate (Days p.i.)	SN Antibody to BDV-M at death
Z999	M/DHxCh*	9	Diarrhoea	Died(22)	7
Z994	F/SxCh	10	Diarrhoea	Died(28)	<5
X730	F/BF	18	Respiratory	Killed(28)	12
V519	F/DH	21	Diarrhoea	Killed(35)	12
102A	F/SxCh	18	None	Killed(273)	73

\* M, Male; F, Female; DH, Dorset Horn; Ch, Cheviot  
S, Suffolk; BF, Blackface

but subsequently seroconverted specifically, having anti-BD titres of 47, 110 and 73 at two, four and eight months after inoculation, respectively, whilst anti-BVD antibodies were undetectable at these times.

Pathology. At necropsy, the 3 lambs that had scoured all had lesions involving the caecum and proximal colon. These lesions varied in intensity from slight thickening to gross oedematous swelling of the bowel wall. The mucous membrane showed diffuse and polypoid hyperplasia, hyperaemia, ulceration and haemorrhage. The lamb with respiratory difficulty had severe encrustation and clogging of the nares. Exudate was present only at the nares, but the nasal mucosa and turbinates were very hyperaemic. This lamb also had thickening of the wall of the caecum and proximal colon.

The distribution of microscopic lesions is given in Table 4.2. In all the affected organs the lesions were essentially lymphoid and proliferative.

The intestinal lesions were most marked in the 3 diarrhoeic sheep. The epithelium was hypertrophied and grossly elongated glands not infrequently breached the muscularis mucosae. The lamina propria was diffusely infiltrated with lymphoid cells and villi were distended. Focal mucosal sloughing with haemorrhage and ulceration was also seen. The submucosa and subserosa were usually oedematous and infiltrated with lymphocytes. Muscle coats were often hypertrophied and oedematous.

In the CNS there were lymphoid infiltrations containing variable proportions of plasma cells and

TABLE 4.2

Experiment 1 - Results of histopathological and virus  
isolation studies from the 4 RHS with clinical disease

Organ	SHEEP NUMBER			
	Z999	Z994	X730	V519
Brain	+/+ *	+/+	+/+	+/+
Large intestine	+/+	+/+	+/+	+/+
Mesenteric Lymph node	+/+	+/+	+/+	+/+
Spleen	+/+	+/+	+/+	+/+
Lung	+ /ND	+/+	+/+	+ /ND
Testis/ovary	- /ND	- /+	- /+	- /+
Liver	- /ND	- /ND	+/+	- /+
Kidney	- /ND	+ /ND	+ /ND	+/+
Heart	+ /ND	- /ND	+ /ND	+ /ND
Thyroid	NE/ND	NE/+	+ /ND	- /+
Submaxillary L.N.	NE/ND	NE/+	NE/+	NE/ND
Blood Clot	NE/ND	NE/+	NE/-	NE/+

\* Histopathology result/ virus isolation result  
e.g. +/+ Lesions present, virus isolated  
NE Organ not examined histologically  
ND Virus isolation not done

macrophages. These were prominent in the choroid plexuses. The choroidal epithelial cells were often rounded and vacuolated. Some had sloughed to form aggregates with lymphocytes in the ventricular system. There was a periventricular encephalitis with perivenous lymphoid cuffs and focal and diffuse glial proliferation. The lamb that recovered had evidence of a residual meningitis when it was killed 273 days after inoculation.

Other organs in which lesions were most often detected were kidney, heart and lung.

Virus isolation. The organs from which virus was isolated are summarised in Table 4.2. Virus was widespread in all animals, being isolated from all samples except the blood clot of X730. All isolates were cytopathic after 3 passages in secondary FLK cells. All were positively identified as BD virus by IFT and selected isolates from each animal were neutralised by specific antisera against BDV-M.

### CONCLUSION

Dramatic and unexpected consequences followed the infection of RHS with a high tissue culture pass of cytopathic BDV-M virus derived from the brain pool with which their dams had been inoculated at 54 days gestation. Profound lymphoid disturbances and widespread virus replication resulted in enteric or upper respiratory tract disease in 4 of the 5 RHS. The development of SN antibodies to the virus by the RHS was feeble in those animals dying or killed in extremis. The surviving animal also seroconverted poorly compared to the controls.

## EXPERIMENT 2

The purpose of this experiment was to repeat the infection of persistently infected lambs with BDV-M and to extend the investigation by infecting similar lambs with BVDV-NADL.

### DESIGN OF EXPERIMENT

Nine persistently infected RHS and four normal sheep, were used. All were free of neutralising antibody to BDV-M and BVDV-NADL viruses, and non-cytopathic BD virus had been isolated from blood clots from all the RHS. The RHS had been born to ewes inoculated at 54 days' gestation with the Moredun IIB brain pool (Barlow, 1972). Three age-matched groups of 3 RHS were housed separately as were 2 groups of 2 normal sheep.

One group (Group 1) of RHS and 1 pair of normal sheep were each injected subcutaneously with 2 ml of a clarified suspension of BDV-M virus grown in FLK cells from the 28th passage virus as described by Vantsis et al., (1980). The suspension, although derived from the same stock and prepared in the same way, was not the same as that used in the first experiment. It contained  $5.2 \log_{10}$  TCID<sub>50</sub> of infectious virus per ml when assayed in tube cultures.

The second group (Group 2) of RHS and pair of normal sheep were each injected subcutaneously with 2 ml of a clarified suspension of BVDV-NADL virus of unknown passage level grown in BEK cells. The inoculum contained  $5.5 \log_{10}$  TCID<sub>50</sub> when assayed in BEK tube cultures.

The third group (Group 3) of RHS was left unchallenged.



Animals were observed clinically, bled for serology 3 weeks after infection and killed for virological examination 4 to 5 weeks after infection.

## RESULTS

Clinical Observations. Clinical symptoms were seen only in 2 RHS in group 1. Animal No. 100A became depressed 16 days after infection and had an intractable scour from 3 weeks onwards. Animal V520 also scoured, but the onset was later and the animal was not so debilitated. The illness in both lambs was severe enough to necessitate killing them on humanitarian grounds. Animals from other groups were killed at the same times for comparative studies.

Serology. Neutralising antibodies to BDV-M and BVDV-NADL at three weeks and time of death are summarised in Table 4.3. Normal susceptible sheep seroconverted to the virus with which they were infected and 3 of 4 of them also produced low levels of antibody to the other pestivirus. All 3 RHS injected with BVDV-NADL but only one of the 3 RHS injected with BDV-M showed evidence of antibody to the viruses they received. None of the RHS produced antibody cross-reacting with the other pestivirus.

Pathology. Gross pathological lesions were seen only in the RHS in group 1. The two animals with scour had lesions in the caecum, proximal colon and terminal ileum similar to those observed in the large intestine of animals in experiment 1. In the third animal (365B) there were focal areas of typhlitis and 1 patch of colitis was seen. In addition, this animal and animal V520 had grey hepatization

TABLE 4.3

Experiment 2 - Serological responses of sheep to  
BDV-M and BVDV-NADL

Group No. Status & Treatment	Animal Number	Sex/ Breed	Age (Months)	Day p.i. Killed	SN antibody at death	
					BDV-M	BVDV-NADL
<hr/>						
Group 1						
Challenged						
with BDV-M						
RHS	100A	M/DHxCh	18	27	<5	<5
RHS	V520	F/DH	30	35	<5	<5
RHS	365B	F/SxCh	8	35	16	<5
Normal	27x84	M/Ch	18	36	335	7
Normal	27x85	M/Ch	18	36	335	5
Group 2						
Challenged						
with BVDV-NADL						
RHS	101A	F/SxCh	18	28	<5	5
RHS	V521	M/DH	30	35	<5	15
RHS	366B	M/SxCh	8	35	<5	25
Normal	27x88	M/Ch	18	ND	9*	35*
Normal	Z197		18	ND	<5*	24*
Group 3						
Unchallenged						
RHS	Z991	M/DHxCh	18	28	<5	<5
RHS	X731	F/BF	30	35	<5	<5
RHS	364B	F/SxCh	8	35	<5	<5

RHS, Recovered Hairy Shaker; M, Male; F, Female

DH, Dorset Horn; Ch, Cheviot; S, Suffolk; BF, Blackface

\* SN antibody levels 35 days post-infection.

in the apical and cardiac lobes of the lungs. The number of tissues showing lesions is summarised in Table 4.4. Lymphoproliferative lesions in the gut, similar to those described in experiment 1, were most severe and widespread in the 2 RHS in group 1 that had scoured, and the other animal in that group. Slight mucosal hypertrophy and focal submucosal and subserosal lymphoid infiltrations were seen in 2 RHS in group 2, and one RHS in group 3.

Lymphoproliferative lesions in other organs were also most severe and were seen most frequently in the RHS in group 1. Mild lesions were present, however, in occasional organs in 1 of the 2 normal animals in group 1, in 2 of the 3 RHS in group 2 and in 2 of the 3 unchallenged sheep in group 3.

Virus isolation. No virus was isolated from any organ from the 2 normal sheep injected with BDV-M. The number of tissues examined and the virus isolation results are summarised in Table 4.4. Of 21 organs tested from unchallenged RHS, 12 contained virus and all were non-cytopathic after 3 weekly passages in FLK cells. Isolates from 2 of the sheep injected with BVDV-NADL were non-cytopathic, but the third animal in this group yielded both non-cytopathic and cytopathic viruses from different organs. Interestingly, this sheep had shown more lesions than the other 2 in the group. In Group 1 RHS, virus was isolated from nearly all the tissues examined. From the 2 animals with clinical symptoms all but one of the isolates were cytopathic. The animal that had not developed disease yielded cytopathic virus from 4 organs but virus from three other organs was not cytopathic.

TABLE 4.4

Experiment 2 - Results of histopathological and virus isolation studies

Group No. Treatment & status	Animal Number	Histopathology No. of tissues examined	No. with lesions	Virus Isolations No. of tissues examined	NCP	CP
<hr/>						
Group 1						
Challenged with BD-M						
RHS	100A	7	7	10	1	7
RHS	V520	6	5	7	1	5
RHS	365B	7	3	8	3	4
Normal	27x84	7	2	6	0	0
Normal	27x85	7	0	6	0	0
Group 2						
Challenged with BVD-NADL						
RHS	101A	7	1	7	4	0
RHS	V521	7	3	7	2	3
RHS	366B	7	0	7	7	0
Group 3						
Unchallenged						
RHS	Z991	7	2	7	5	0
RHS	X731	7	0	7	3	0
RHS	364B	7	2	7	4	0

NCP, Non-cytopathic virus; CP, Cytopathic virus

RHS, Recovered hairy shaker

## DISCUSSION

In two experiments, cytopathic virus derived from the 28th passage of BDV-M produced disease in a total of 7 of 8 RHS. Six of these animals died or were killed on humanitarian grounds. These animals, and one of the remaining two which was killed 35 days post-infection had widespread and severe lymphoid proliferations in several organs. No disease was seen in any of the other sheep, but minor lesions of the same type were present in occasional organs of one of two normal susceptible sheep receiving the same inoculum, as well as in 2 of 3 RHS receiving BVDV-NADL and in 2 of 3 uninoculated RHS. There was, thus, strong evidence that the high passage BDV-M inoculum was pathogenic for RHS, although the possibility that the RHS were uniquely susceptible to some other unknown infection cannot be totally discounted.

Disseminated lymphoid proliferations of the type seen here occur in some neoplastic disorders and in conditions such as bovine malignant catarrhal fever (MCF). Major features of MCF lesions, namely focal necrosis of lymph nodes and fibrinoid necrosis of the media of arteries, appeared to be absent in these sheep, however.

The CNS lesions were similar to those described following maedi/visna virus infection of sheep (Palsson, 1976). Serological examination of the sheep in the present experiments showed that they were all free of precipitating antibody to this virus (Dawson, personal communication). The lung changes seen were similar to those occurring in maedi, but they have also been

demonstrated in cases of chronic atypical ovine pneumonia (Gilmour, Jones and Rae, 1979). Since the latter is a common disease of sheep the possibility that the pulmonary changes represented intercurrent infection cannot be excluded.

The enteric lesions were more specific, being histologically distinguishable from most other forms of ovine enteritis. Some of the changes, namely the hyperplastic epithelium and the glandular penetration of the submucosa with extension into lymphatics, were tumour-like and closely resembled those seen in porcine adenomatosis (Rowland, lawson and Maxwell, 1973). Others, such as the ulcerated, haemorrhagic, heavily infiltrated hyperplastic mucosa with crypt abscesses, strongly resembled lesions seen in calves with mucosal disease. Furthermore, the distribution of the enteric lesions present in affected sheep was similar to that of the most marked pathomorphological changes seen in cattle with mucosal disease (Ohmann, 1983).

The purpose of these experiments was to see if RHS, persistently infected with non-cytopathic BD virus, were immunocompetent when challenged with cytopathic homologous (BDV-M) or heterologous (BVDV-NADL) pestiviruses. The widespread and severe lymphoproliferative lesions in the RHS injected with homologous BDV-M suggested an immunological response. Cytopathic virus was not eliminated, however, and the animals failed to produce convincing levels of specific neutralising antibody when compared to the normal susceptible sheep receiving the same

inoculum. Their immune system, therefore, appeared compromised when faced with this virus, possibly due to tolerance resulting from exposure to the same virus during early foetal life. If this was the case, the tolerance was not absolute since in the one surviving animal specific SN antibodies were produced, albeit slowly and to a relatively low titre. Even though it survived for 10 months this animal did not develop cross-neutralising antibody against BVDV-NADL.

The BVDV-NADL inoculum was weakly immunogenic as judged by the low levels of neutralising antibody in the 2 normal susceptible sheep. Nevertheless, all 3 RHS injected with this virus seroconverted to it and remained healthy, cytopathic virus having been largely eliminated. Although lack of disease may have been due to low pathogenicity of the virus resulting from prolonged passage in tissue culture, these findings suggest a competent immune response to the virus.

If immune tolerance is responsible for virus persistence, these results suggest that the tolerance is highly specific. Analogous findings have been reported in cattle. Steck et al.(1980) immunised 209 seronegative animals with an attenuated live virus vaccine, prepared from the BVDV-C24V strain. Two hundred and seven seroconverted. The two seronegative animals became clinically ill 8 and 16 days post-vaccination, and MD was diagnosed in the first of these. Four other animals died from a disease indistinguishable from MD, even though, after vaccination, they developed high titre SN antibody



which was directed specifically against C24V strain virus and which did not react with the virus involved in the persistent viraemia. This latter virus was neutralised by sera from other healthy animals in the herd, but not by sera from the dying animals. A very specific immune dysfunction is implied by these results.

The results of the experiments reported in this Chapter revealed a new aspect of BDV infection of sheep. The enteric lesions resembled changes seen in calves with mucosal disease, thus establishing a further link between BDV and BVDV infections in the two species. The mucosal disease-like syndrome in sheep was restricted to RHS receiving the BD-M inoculum, suggesting that the antigenic relationship between the persistently infecting and the 'superinfecting' virus was an important factor in the pathogenesis of the disease. One other unknown factor, however, was the relative pathogenicities of the two high passage viruses. It, thus, seemed important to characterise virus stocks of known passage history, and this work is described in the next Chapter.

## CHAPTER 5

### PATHOGENICITY STUDIES WITH LOW PASSAGE PESTIVIRUSES

#### INTRODUCTION

The pathogenicity of pestiviruses has been shown to change after passage in-vivo and in-vitro. During early transmission experiments with BVD virus it had been noted that clinical signs in calves were more intense after inoculation with a recently isolated BVD virus than with the same virus after it had been passaged in calves 4 to 6 times (Pritchard,1963). Similarly, the passage of BVD virus strains in tissue culture cells has been shown to decrease their pathogenicity for calves (Coggins et al.,1961; Castrucci, cited in Roeder et al.,1983).

The use of BVD viruses passaged more than 20 times in bovine tissue cultures has made it difficult to interpret the results of some experimental infections of calves. Both attenuation of virulence of the viruses, and their possible contamination with non-cytopathic pestiviruses derived from cell cultures or serum supplements have had to be considered (Nuttall et al.,1980; Lopez et al., 1982).

The same considerations need to be applied to studies with high passage BD viruses. The mechanisms involved in the new and interesting lymphoproliferative disease reported in the last Chapter require elucidation. This will be achieved with a high degree of certainty only if low passage virus free of adventitious contamination with non-cytopathic pestiviruses is used.

In this Chapter, experimental infections of sheep and cattle with low passage BVDV-NADL and BDV-M are described. They were undertaken to study the pathogenicity of the strains and to prepare reference antisera.

SECTION 1. REFERENCE ANTISERUM PRODUCTION AND PATHOGENICITY STUDIES WITH LOW PASSAGE BVDV-NADL VIRUS.

Antisera prepared against BVD viruses have been shown to react with components of uninfected cells in CF, ID and IF tests. The problem has been most frequently associated with hyperimmune sera prepared in rabbits but sera from sheep and cattle have also suffered from this disadvantage (Fernelius and Packer, 1969; Hassan and Scott, 1980). In an attempt to abrogate this difficulty antisera to BVDV-NADL were prepared in calves using autologous tissue cultures and serum supplements.

MATERIALS AND METHODS

Animals. Two Jersey bull calves were used. At two months of age they were bled and shown to be free of serum neutralising antibody to BVDV-NADL and BDV-M viruses. One month later, approximately 200 ml. of serum was derived aseptically from each calf. Three days later the calves were castrated under general anaesthesia. They recovered well from the operation.

Cell culture and virus growth. Primary testis cultures were prepared by trypsin disaggregation. Cultures from each calf were handled separately and grown at 37°C in Eagle's MEM supplemented with 10% of the calves' own sera.

When confluent, cell monolayers were washed three times with Hanks' BSS and inoculated with low passage BVDV-NADL at a m.o.i. of one. After 2 hours' adsorption at 37°C, the inocula were removed and maintenance medium of Eagle's MEM supplemented with 2% of the calves' own sera added. Three days later cultures showed extensive CPE. Supernatant fluid was harvested, clarified at 2000 xg for 15 minutes at 4°C and divided into 4 ml. aliquots, which were stored at -70°C. The titre of virus from each calf was  $7.2 \text{ Log}_{10} \text{TCID}_{50}$  per ml. when they were assayed in tube cultures of autologous cells.

Animal inoculation. Twelve days after castration each calf was injected intravenously with 1 ml. of virus grown in its own cells. Two weeks, three weeks and four weeks later each calf was injected intravenously with 4 ml., 5 ml. and 10 ml. of the same virus harvests. Two weeks after the last injection calves were bled out, and separated serum was stored at -20°C in 5 ml. amounts.

Pathogenicity studies. Clinical examination of the calves, including temperature recording, was carried out daily for 3 weeks after injection. Calves were bled, daily for 3 weeks and at weekly intervals thereafter, into heparinised vacutainer tubes for haematological studies and into vacutainer tubes for serological studies.

Haematology. This was performed as described in Chapter 2.

Serology. Sera were heat inactivated, and titrated for the measurement of neutralising antibodies to BVDV-NADL and BDV-M as described in Chapter 2. Dilutions of sera were used in the first stage of an IIFT against uninfected and BVDV-NADL infected BEK cells.

## RESULTS

Clinical observations. Both animals showed mild clinical symptoms of diarrhoea and serous nasal discharge, 5 to 8 days after inoculation with virus. Calf 368 was anorexic on days 6 and 7. Both calves had pyrexia and leucopaenia, although the degree and timing of these events were different (Fig. 5.1). Calf 368 showed a biphasic temperature response with the first peak being detectable on days 6 and 7, and the second peak on day 10. Calf 385, however showed evidence of pyrexia from day 2 onwards with values  $> 40^{\circ}\text{C}$  on days 4, 5, 8 and 11.

The leucopaenia was most pronounced in calf 368 with cell counts being depressed between day 2 and day 12 by  $> 25\%$  of the level at the time of infection. Calf 385 had leucopaenia from day 4 to day 6 and leucocytosis on day 8. Differential leucocyte counts showed the leucopaenia to be due to a decrease in both lymphocytes and neutrophils, although the neutropaenia was particularly marked in both calves between 4 and 6 days after infection (Table 5.1).

Serology. Serum neutralising antibodies to BVDV-NADL virus were first detected at a low level 11 days after infection. Thereafter, antibody levels rose quickly until day 17, but continued to rise until the final bleeding on day 45 when sera from both calves had SN titres of 2560 (Figure 5.2). Neutralising antibody titres to BDV-M at the final bleeding were 80 and 40 respectively for calves 385 and 368. No cytotoxicity was observed in the SN tests at reciprocal serum dilutions of 4.

FIGURE 5.1      The clinical and haematological responses of  
2 Jersey calves to the intravenous injection  
of  $7.2 \log_{10} \text{TCID}_{50}$  of low passage BVDV-NADL.

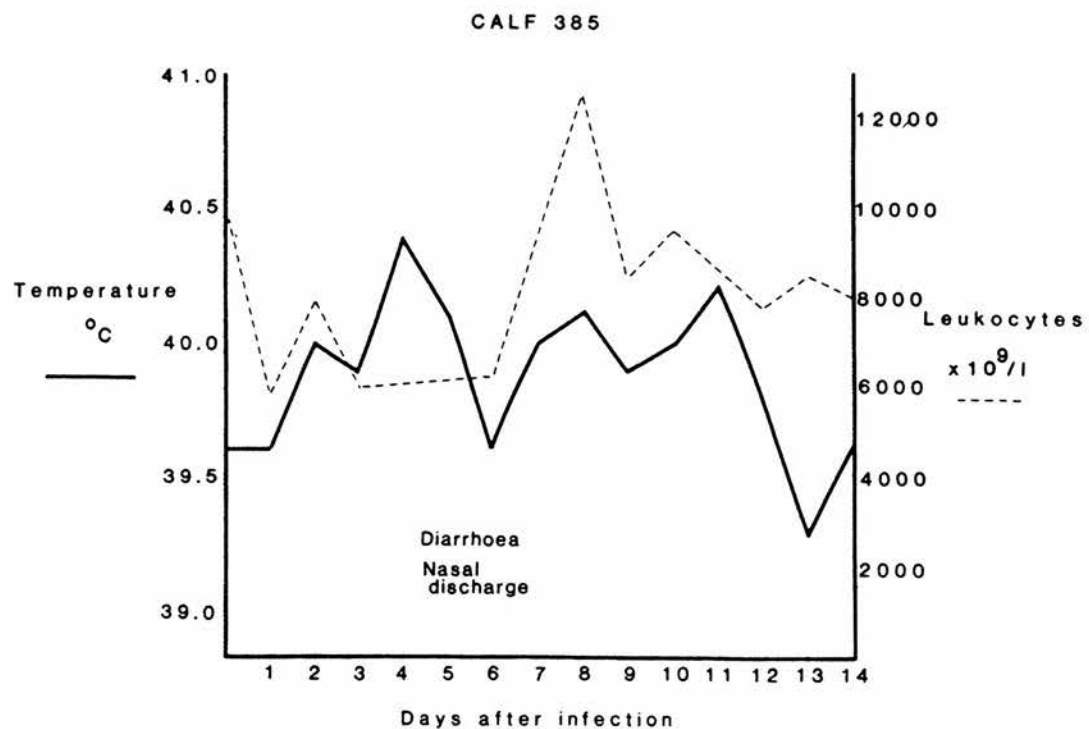
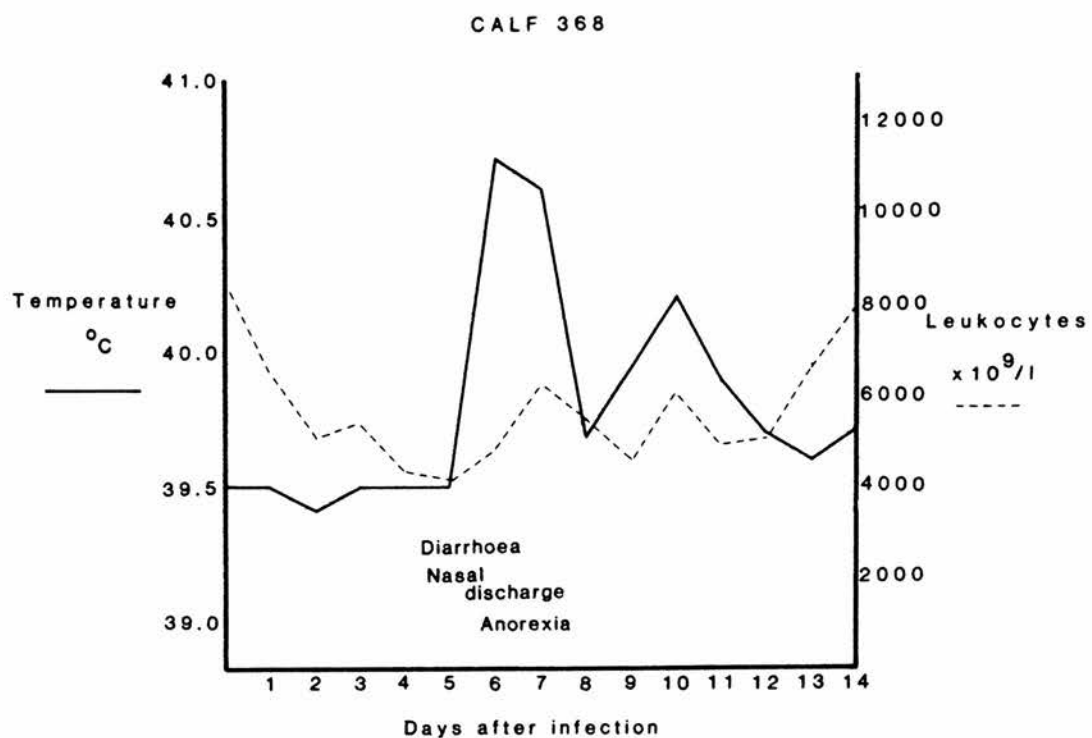




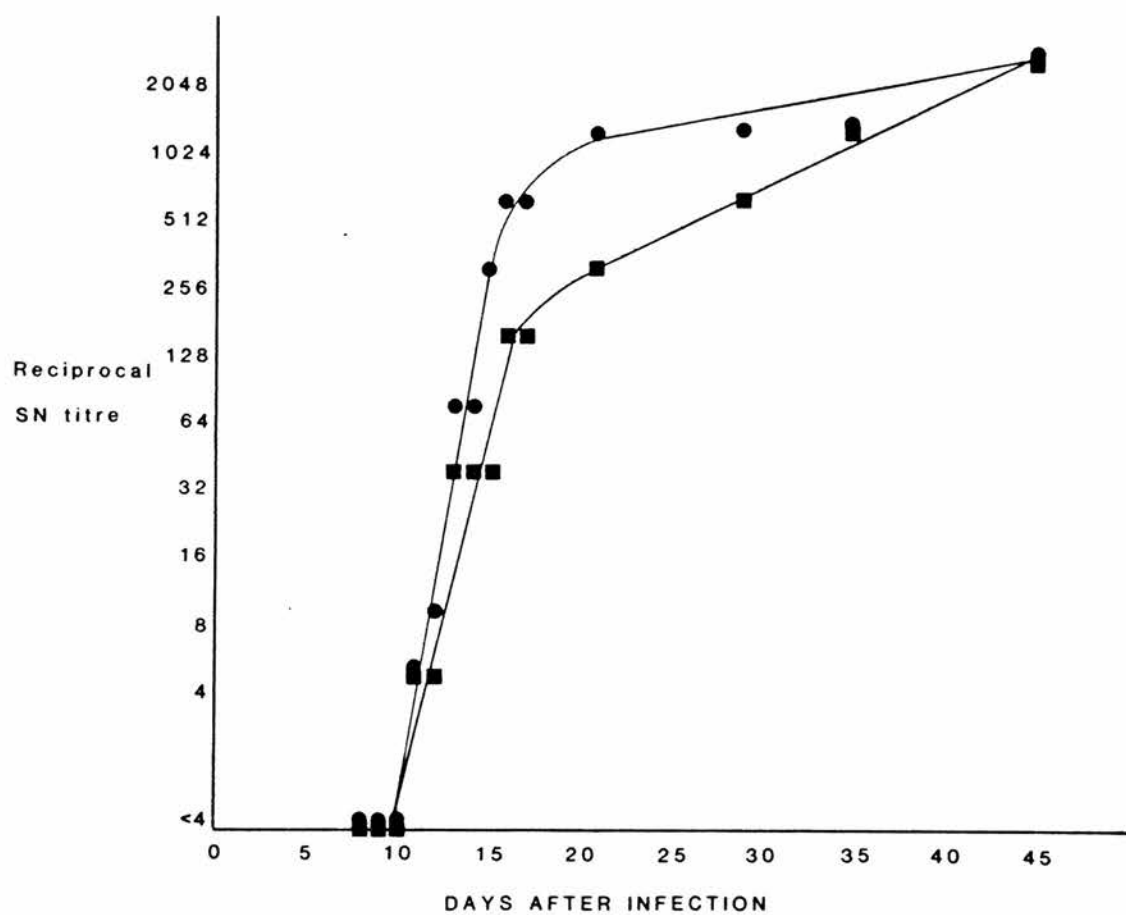
TABLE 5.1  
Differential leucocyte counts for 2 calves  
infected with low passage BVD-NADL virus

Days after Infection	Mononuclear leucocytes		Polymorphonuclear leucocytes	
	Calf 368	Calf 385	Calf 368	Calf 385
0	6043(72.5)*	7961(81.0)	2292(27.5)	1867(19.0)
1	5622(86.0)	4670(83.0)	915(14.0)	957(17.0)
2	4393(85.0)	5927(74.5)	775(15.0)	2029(25.5)
3	4866(89.0)	5446(86.5)	601(11.0)	800(13.5)
4	4181(97.0)	5997(96.5)	129( 3.0)	217( 3.5)
5	4040(94.5)	5394(86.5)	235( 5.5)	842(13.5)
6	4667(97.0)	5919(93.5)	144( 3.0)	411( 6.5)
7	5091(82.0)	7872(87.0)	1117(18.0)	1176(13.0)
8	5021(90.0)	11655(90.0)	558(10.0)	1295(10.0)
9	4203(91.5)	6632(78.0)	390( 8.5)	1870(22.0)
10	5748(93.0)	7520(80.0)	433( 7.0)	1880(20.0)
11	4541(92.0)	6292(76.0)	395( 8.0)	1982(24.0)
12	4583(91.5)	6450(82.0)	426( 8.5)	1416(18.0)
13	5485(81.5)	6439(75.5)	1245(18.5)	2090(24.5)
14	6757(85.5)	6454(77.5)	1146(14.5)	1874(22.5)

\* Results are expressed as absolute counts  $\times 10^9/l$  and the percentage of the total leucocyte count is shown in brackets.

FIGURE 5.2      The development of serum neutralising antibodies to BVDV-NADL by two Jersey calves. The calves were injected intravenously on day 0, day 14, day 21 and day 28 with 1ml, 4ml, 5ml and 10ml respectively, of low passage BVDV-NADL, containing  $7.2 \log_{10}$  TCID<sub>50</sub> per ml.

■ — ■ Calf 368      ● — ● Calf 385



In the IIFT, no specific fluorescence was detected in uninfected cells exposed to the calf sera diluted 1:10. Infected cells, however, fluoresced brightly up to serum dilutions of 1:40, and weak fluorescence was discernible up to dilutions of 1:160.

### CONCLUSIONS

Low passage BVDV-NADL was pathogenic for both calves producing diarrhoea, pyrexia and leucopaenia, principally neutropaenia. These findings were consistent with previously reported experimental infections (Pritchard, 1963). High titre specific neutralising antibodies were produced which cross-reacted with BDV-M virus, but which were free of antibody to uninfected bovine cells.

### SECTION 2. Reference antiserum production and pathogenicity studies with low passage BDV-M, following the infection of ewes in late gestation.

Lambs infected with BD virus in-utero during late gestation are born with specific SN antibodies to the virus (Gardiner, 1982). This section describes the exploitation of this ability of BD virus to produce antisera free of non-specific reactions.

### MATERIALS AND METHODS

Experimental procedure. Four seronegative Blackface ewes were injected intravenously at 115 days' gestation with 2 ml. of low passage BDV-M containing  $6.4 \text{ Log}_{10} \text{ TCID}_{50}$ . Lambs

were to be delivered into sterile isolators and reinfected intranasally with the same dose of virus two days after birth. Lambs put into isolators were exsanguinated under general anaesthesia at three weeks of age, and brain and spinal cord collected into 10% formal saline for pathological examination.

Four seronegative, castrated male Blackface lambs were injected subcutaneously with 2 ml of tissue culture fluid derived from uninfected cells passaged in parallel with those used to grow BDV-M virus since its derivation from the IIB brain pool.

Serology. Sera were heat inactivated, and titrated for the measurement of SN antibodies to BVDV-NADL and BDV-M as described in Chapter 2. Dilutions of sera were used in the first stage of an IIFT against uninfected and BDV-M infected FLK cells.

### Results

Outcome of infecting ewes. Twenty eight days after infection 2 ewes produced apparently normal single lambs. The lambings were unattended, both lambs drank colostrum and were not used further. One ewe lambed naturally 31 days after infection; the lambing was attended and the lamb transferred to an isolator. Twin lambs from the fourth ewe were delivered by caesarian section 33 days after infection and reared together in one isolator. The lambs in the isolators were never strong and the single lamb had superior prognathism from birth. No specific symptoms developed following intranasal infection with BDV-M.

Serology. All four ewes had produced high antibody titres to BDV-M (>360) when bled 21 days after infection. The control lambs given tissue culture fluid had no detectable SN antibody to BDV-M or BVDV-NADL viruses one month after injection.

Lambs born to infected ewes all had low SN antibody titres to BDV-M at birth namely 3, 22 and 180 respectively; and these were raised to 1440, 256 and 720 at death. Serum neutralising antibody titres against BVDV-NADL at the final bleedings were 8, 16 and 8 respectively. No cytotoxicity was observed in the SN tests at dilutions of 1:4. Sera from the final bleedings showed no specific fluorescence at dilutions of 1:10 when tested against uninfected FLK cells in an IIFT. Against infected cells, however, these sera gave bright, specific intracytoplasmic fluorescence up to dilutions of 1:40, with less obvious fluorescence at dilutions of 1:80 to 1:160.

Pathology. Lesions of periarteritis were detected in the CNS of the three lambs.

### CONCLUSIONS

Specific antisera to BDV-M had been raised. This had low neutralising activity against BVDV-NADL and did not react with uninfected FLK cells. The CNS lesions were similar to those seen in lambs born to ewes infected late in gestation with the Moredun IIB brain pool (Zakarian et al., 1975).

### SECTION 3. Protection test between BVDV-NADL and BDV-M in ewes.

Results in the previous two sections had demonstrated a low degree of relatedness between BDVV-NADL and BDV-M in reciprocal neutralisation tests. The experiment described in this section was designed to see if exposure of ewes before pregnancy with BVDV-NADL would protect, or change the pathogenesis of disease in their progeny following challenge of the ewes at 50 days gestation with BDV-M virus. An earlier study, in which 6 ewes previously exposed to BVDV-NADL were challenged with Moredun IIB pool, had resulted in a range of foetal abnormalities; one ewe aborted, one produced a BD-affected lamb, one produced a lamb with severe neural and skeletal malformations (AP), and three had normal lambs (Barlow et al., 1980a).

#### MATERIALS AND METHODS

Animals and experimental procedure. Eighteen mature ewes without SN antibodies to BVDV-NADL and BDV-M were used. Twelve ewes were separated from the other 6 and injected subcutaneously with 2 ml of low passage BVDV-NADL virus containing  $4.0 \log_{10} \text{TCID}_{50}$ .

The ewes were oestrus-synchronised and 1 month after infection a tup was added to each group. Seven weeks later, between 49 and 51 days' gestation, the ewes were bled and arranged into groups as summarised in Table 5.2. At this time all animals except those in group 5 were injected subcutaneously with 2 ml of low passage BDV-M virus containing  $6.4 \log_{10} \text{TCID}_{50}$ . At 75 or 85 days' gestation, the ewes were bled and their uteri collected for



TABLE 5.2

Reciprocal serum neutralising antibody titres of the ewes  
in a protection test between low passage pestiviruses

Group No.	Viruses received	Ewe No.	SN antibody Week 11		Week Killed	SN antibody at death	
			BVD NADL	BD-M		BVD NADL	BD-M
1	BVD-NADL	V975	90	<4	14*	360	256
	(Week 0)	N632	90	6	14	>720	>720
	BD-M	N616	180	4	14	>720	720
	(Week 11)	X972	256	6	14	>720	720
		T655	64	<4	14	>720	720
2	BVD-NADL	V998	45	<4	16+	>720	360
	(Week 0)	387A	128	6	16	180	90
	BD-M	406A	256	6	16	360	256
	(Week 11)	289B	128	<4	16	180	180
		T656	180	<4	16	720	360
3	BVD-NADL	350A	360	8	14	45	4
	(Week 0)	V982	64	<4	14	256	<4
4		Z791	<4	<4	14	ND	256
	BD-M	X253	<4	<4	14	ND	180
	(Week 11)	X298	<4	<4	16	ND	360
		T550	<4	<4	16	ND	128
5	NONE	X182	<4	<4	14	<4	<4
		X262	<4	<4	16	<4	<4

\* Week 14 represented 75 day's gestation.

+ Week 16 represented 85 day's gestation.

virological and pathological studies of the placentae and fetuses.

Serology. Serum samples from ewes and fetuses were tested for the presence of SN antibodies to low passage BVDV-NADL and BDV-M as described in Chapter 2.

Virology. For virus isolation the following samples were collected. Cotyledon, blood clot from all fetuses, cerebellum from second and third fetuses. Samples were tested for the presence of viruses, using a minimum of 3 passages at weekly intervals in secondary FLK cells, as described in Chapter 2.

Pathology. Fetuses and placentae were perfused with Baker's calcium formol or Bouin's fixative. The methods used for the examination of tissue sections, and the criteria selected for the classification of the CNS lesions, were as described previously (Barlow, 1980; Barlow et al., 1980a).

## RESULTS

All the ewes injected with BVDV-NADL developed SN antibody to the virus and 6 of the 12 developed low levels of cross-neutralising antibody to BDV-M. Following challenge with BDV-M, the 10 ewes in groups 1 and 2 developed high SN antibodies to both viruses. Animals in the other 3 groups, which received one or other of the viruses, produced good SN titres to the virus with which they were infected (Table 5.2).

Injection with BVDV-NADL 4 weeks before pregnancy protected the progeny of only one of the 10 ewes (X972 in group 1) challenged at 50 days' gestation with low passage BDV-M (Table 5.3).

TABLE 5.3

Virological and pathological findings in placenta and  
foetuses from ewes used in a protection test  
with low passage pestiviruses

GROUP No.	EWE No.	VIRUS ISOLATION				PATHOLOGY	
		Plac- enta	1st foetus Blood	2nd foetus Blood	CNS	Plac- enta	Foetal CNS
1	V975	---	NCP	NCP	NCP	---	---
	N632	NCP	NCP	NCP	---	---	BD
	N616	---	NCP	NCP	NCP*	BD	BD
	X972	---	---	---	---	---	---
	T655	---	NCP	NCP	NCP	BD	BD
2	V998	---	NCP	No foetus		---	BD
	387A	NCP	CP	NCP	NCP	---	BD
	406A	NCP	CP	CP	CP	BD	BD
	289B	---	NCP	NCP	NS	BD	BD
	T656	---	NCP	NCP	NCP	BD	BD
3	350A	---	---	---	---	---	---
	V982	---	---	---	---	---	---
4	Z791	NCP	NCP	CP	NCP	BD	BD
	X253	NCP	CP	NCP	NCP	BD	BD
	X298	CP	CP	---	---	BD	BD
	T550	NCP	---	No foetus		BD	NS
5	X182	---	---	No foetus		---	---
	X262	---	---	No foetus		---	---

\* Three foetuses were tested from ewe no. N616. Noncytopathic BDV was also isolated from the blood and CNS of the third foetus.

---, No virus isolated or no pathological evidence of BD

NCP, Non-cytopathic BDV isolated

CP, Cytopathic BDV isolated      NS, No sample

BD, Pathological evidence of BD

There was no significant difference ( $\chi^2 = 0.1$ ,  $p > 0.05$ ) between the number of BD virus isolates from ewes previously exposed to BVDV-NADL (Groups 1 and 2) and those that were not (Group 4). Some qualitative differences in virus isolations were, however, apparent between these groups. Virus was recovered less readily from the placenta of the ewes from groups 1 and 2 compared with group 4. Cytopathic BDV-M was not recovered from ewes in group 1, and was isolated from only the foetuses of 2 ewes in group 2. In group 4, however, cytopathic virus was recovered from progeny of 3 of 4 ewes and from the placenta of one of these animals.

Pathological lesions were consistent with a diagnosis of BD. No evidence of AP was seen in any of the foetuses. The effects of BDV-M on the uterine contents were obvious grossly in 3 of the ewes in group 4. Both ewes killed at 75 days gestation had hydrops amnii; foetuses were alive but deeply congested and one had anasarca. The foetus of ewe T550 was dead and unfit for pathological examination. Gross pathological lesions were not detected in ewes from groups 1 and 2, but the presence of BD-associated lesions in the placenta and foetal CNS correlated closely with the results of virus isolation (Table 5.3).

No placental or foetal lesions were seen in the control animals in groups 3 and 5, nor was any virus detected in samples collected from these animals.

## CONCLUSIONS

The progeny of ewes injected with BVDV-NADL 1 month before pregnancy were not protected from BDV-M virus when the ewes were challenged at 50 days' gestation. There was some evidence, however, that the prior exposure of ewes to BVDV-NADL reduced the severity of the BD-specific placental and foetal lesions.

## SECTION 4. PATHOGENICITY TESTING OF LOW PASSAGE BDV-M BY INFECTION OF EWES AT 54 DAYS' GESTATION.

Moreduin IIB brain pool has consistently produced lambs with classical BD pathology when injected into pregnant ewes at 54 days' gestation (Gardiner, 1982). When, however, tissue culture grown virus derived from this pool was injected into ewes at titres of 5.0 and 6.8  $\log_{10}$ TCID<sub>50</sub> all but one of 39 sheep aborted (Vantsis et al., 1976). The results from the previous section showed that the inoculum used in that experiment was also very pathogenic for foetuses. Using tissue homogenates, Richardson et al., (1976) demonstrated that more live lambs were born to ewes receiving a lower dose of BD virus. This section describes the production of BD lambs using low passage BDV-M, and their survival in the first seven months of life.

## MATERIALS AND METHODS

Inoculation of pregnant ewes. A group of Cheviot ewes from a farm with no history of BD and without SN antibody to BDV-M or BVDV-NADL were oestrus-synchronised and mated to

two Dorset Horn rams, which were also seronegative to both viruses. On the 54th day of gestation 15 ewes were housed and injected with 6 ml of tissue culture fluid containing  $3.0 \text{ Log}_{10} \text{TCID}_{50}$  per ml of low passage BDV-M. Half the dose was given subcutaneously and half intraperitoneally.

Monitoring and sampling of ewes and lambs. All ewes were bled one month after injection with virus to assess their SN antibody status to BDV-M. The ewes were examined daily; animals that aborted, were found to be barren, or produced dead lambs were removed from the group. Live lambs were bled for virus isolation and SN antibody determination, before being given colostrum from their dams. Lambs that could stand were encouraged to feed from their dams, but those that were too weak were hand reared on artificial milk. Lambs were bled at regular intervals throughout life for virological and serological studies. Lamb illnesses and treatments were recorded.

## RESULTS

Response of ewes to infection with low passage BDV-M. No ewe showed any evidence of malaise after infection. All ewes seroconverted to the virus, producing SN titres in the range 180 to 2048. Five ewes aborted between 99 and 140 days gestation, three ewes produced stillborn lambs at term, two were barren, one ewe produced one stillborn and one live lamb, and the other four ewes each produced live BD twins.

Of the 15 ewes, 5 were mated by one tup and 10 were mated by the other. Only one of the 5 ewes mated by the first tup produced live lambs (Twins - 1261 and 1262), the other 7 live lambs being born to 4 ewes mated by the second tup.

Virology and serology of ewe's progeny. Virus isolation was attempted from brain/spleen pools from the aborted foetuses of three ewes; NCP BD virus was isolated from one of twins from one ewe. The same type of virus was isolated from two stillborn lambs, but a cytopathic BD virus was recovered from a tissue pool of a third. None of the nine live lambs had detectable SN titres to BDV-M at birth, but NCP BD virus was recovered from blood clots of all of them.

Six weeks after birth 5 lambs had detectable SN antibody to BDV-M, and this number had fallen to 4 two weeks later. One week after this second bleeding, two seronegative lambs were killed in-extremis (Twins - 1261 and 1262 (see below)). The remaining 7 lambs were seronegative and viraemic 10 weeks after birth.

Disease among BD lambs. At birth, all lambs had characteristic nervous signs of BD, but only 2 lambs required artificial feeding (1250, 1254); two weeks after birth these lambs were able to stand unaided.

One month after birth, one lamb (1251) was depressed, mildly pyrexia (40.5°C) and hyperpnoeic; pneumonia was diagnosed and daily treatment for 5 days with Trivetrin injection (Wellcome Foundation Ltd.) effected recovery.

Six weeks after birth, two lambs (Twins - 1261 and 1262) were first seen to be depressed. Over the next 18 days Lamb number 1261 developed an intractable scour which was not alleviated by treatment with Kaogel (Parke Davis & Co.) and Ion-aid (Syntex Pharmaceuticals Ltd.). Lamb 1262 did not scour. Both lambs became very depressed, and developed mucopurulent nasal and ocular discharges; lamb



1262 showed bilateral corneal opacity (Figure 5.3), and both lambs were killed on humanitarian grounds three weeks after first becoming ill. Haematological examination showed that these two lambs had lymphocytopaenia and neutrophilia, 6 and 8 weeks after birth, compared with the other lambs (Table 5.4). Cytopathic BD virus was recovered from the buffy coats of both lambs collected just before death.

At necropsy, both lambs had gross thickening of the distal ileum, caecum and colon. This was the result of a focal hyperplastic enteropathy, which was most apparent in transverse gut sections or when the mucosal surface was exposed (Figures 5.4 and 5.5). Small shallow mucosal ulcers were present in lamb 1261 but not lamb 1262. Histologically, however, both lambs showed ulcerative hyperplasia of the mucosa with foci of massive penetration of the muscularis, around which there was mononuclear cell infiltration and necrosis. Generally, though, there was relatively little lymphoid infiltration of the gut or other organs. The thymus in both lambs was atrophic, and the lymph nodes and spleens showed poor follicle development. In the CNS, both lambs had residual lesions of BD.

Cytopathic BD virus was recovered from 15 of 16 organs cultured. No bacteria were cultured from the spleens and only E.coli and Proteus species were isolated from the large intestine.

The remaining 7 lambs all scoured due to coccidiosis at 12 weeks of age, but remained bright and responded well to treatment with Amprol-plus (Merck Sharp & Dohme Ltd.).

Figure 5.3      Lamb 1262, three weeks after first becoming ill. Nasal and ocular discharges, and excess salivation have resulted in the matting of wool and scab formation around the eye and muzzle. The degree of corneal opacity is greatly exaggerated in this photograph.

Comparison with Figure 1.5 is interesting.

Figure 5.4      Lamb 1262. Transverse section of colon showing hyperplastic enteropathy with pale tissue bulging from cut surface.

Figure 5.5      Lamb 1262. Mucosal surface of colon. Raised rounded areas of thickened gut wall are prominent.



TABLE 5.4

Haematological values of border disease lambs  
six and eight weeks after birth

Lamb No.	Weeks after birth	Erythrocytes	Haemoglobin g/dl	LEUCOCYTES		
				Total	Mono-nuclear	Polymorpho-nuclear
1250	6	9.07*	9.7	6.69+	3.68(55)	3.01(45)
	8	10.35	10.5	8.61	5.34(62)	3.27(38)
1251	6	10.89	11.9	4.09	3.51(86)	0.57(14)
	8	11.49	12.4	7.56	6.58(87)	0.98(13)
1254	6	8.91	9.0	7.22	3.03(42)	4.19(58)
	8	8.44	7.9	3.70	2.30(62)	1.40(38)
1255	6	10.94	12.2	4.47	4.16(93)	0.31 (7)
	8	11.87	12.8	10.70	9.63(90)	1.07(10)
1256	6	9.30	10.5	4.21	3.87(92)	0.34 (8)
	8	11.09	11.5	7.06	5.72(81)	1.34(19)
1257	6	10.87	11.2	6.16	5.61(91)	0.55 (9)
	8	13.21	12.1	9.03	7.77(86)	1.26(14)
1259	6	9.42	10.8	5.79	5.16(89)	0.64(11)
	8	11.23	12.7	4.71	4.28(91)	0.43 (9)
1261	6	10.47	10.4	10.40	7.59(73)	2.81(27)
	8	12.25	14.1	11.30	1.58(14)	9.72(86)
1262	6	10.59	12.3	4.37	2.01(46)	2.36(54)
	8	11.60	11.9	5.74	1.89(33)	3.84(67)

\* Erythrocyte counts are given  $\times 10^{12}/l$

+ Total leucocyte counts are given  $\times 10^9/l$

Differential leucocyte counts are also given  $\times 10^9/l$   
and the % of the total count is shown in brackets

One lamb died suddenly from acute pneumonia at five months old. Pasteurella haemolytica type A2 and Mycoplasma ovipneumoniae were cultured from the lungs. Non-cytopathic BD virus was isolated from 7 of 8 organs cultured.

The remaining 6 lambs grew poorly, but suffered no other major illnesses. At 7 months old they were used in another experiment.

CONCLUSION. Low passage BDV-M was pathogenic for the foetuses of ewes inoculated at 54 days' gestation. Only 9 live lambs were produced by 5 of 15 ewes. Two of the lambs died at 9 weeks of age, with disease very similar to the mucosal disease-like syndrome described in the experimentally superinfected ewes (Chapter 4), and one lamb died of pneumonia at 5 months old.

#### DISCUSSION

Work reported in this Chapter has confirmed the pathogenicity of the low passage stocks of the two pestivirus reference strains. The BVDV-NADL strain was isolated originally from a fatal case of MD in a closed herd (Gutekunst & Malmquist, 1963). It has been reported frequently that BVDV isolates from fatal cases produce only mild disease when injected into susceptible cattle (Baker et al., 1954; Dow et al., 1956; Huck, 1957; Thomson and Savan, 1963; Pritchard, 1963). The pyrexia, leucopaenia, diarrhoea, and nasal discharges produced in the two calves, used to prepare specific antisera, were wholly comparable to the range of symptoms described by these earlier authors.

Little information is available on the pathogenicity of BD virus for normal susceptible sheep, and so pregnant animals were used. Infection of ewes in late gestation resulted in the birth of lambs with low levels of SN antibody, and with residual CNS lesions of periarteritis. These confirmed the placenta-crossing ability of the virus and agree with the pathological findings in lambs from infections initiated between 90 days' gestation and early post-natal life (Zakarian et al.,1975).

Infection of ewes at 54 days' gestation further confirmed the pathogenicity of the BDV-M virus. Previous exposure of the ewes to BVDV-NADL virus did not protect their progeny, which showed lesions of hypomyelinogenesis and interfascicular lipid consistent with a diagnosis of BD. This contrasts with previous reports in which IIB pool produced AP in a proportion of ewes infected previously with BVDV-NADL, and also in a proportion of ewes injected with IIB pool which had been incubated in-vitro with antiserum against BVDV-NADL (Barlow et al.,1980a; Gardiner,1982). The difference could have been due to the large challenge dose of BDV-M used in the experiment reported here.

Even a much reduced challenge dose was highly pathogenic for the progeny of susceptible ewes. It has been reported previously that the genotype of the dam can influence the outcome of foetal infection (Barlow et al.,1979; Barlow et al.,1980b; Barlow,1980). In this experiment there was some evidence that the the genotype of the sire could have influenced the outcome. Four of ten

ewes sired by one ram produced live lambs, whereas only one of 5 ewes sired by the second ram gave birth to live lambs. This, in itself, is not significant, but it was interesting that only the twins born to this ewe later succumbed to BDV-related disease. The disease seen in these two lambs bore a strong resemblance to the mucosal disease-like syndrome described in Chapter 4, and provided further evidence for the spontaneous occurrence of such cases as reported previously (Barlow et al., 1983). The recovery of cytopathic BDV from these two lambs was also consistent with the finding in previous spontaneous and experimental cases (Gardiner et al., 1983). At birth only NCP virus had been recovered from the blood of both these lambs, and the onset of disease coincided with loss of maternally-derived antibody. This apparent reassertion of pathogenicity by BDV, correlating with its ability to produce CPE in cell cultures, was associated with profound haematological changes in the twin lambs compared with their contemporaries. It was also noteworthy that the disease was not transmitted from the twins to their contemporaries with which they were housed.

Cell culture-derived cytopathic BDV has previously been shown to cross the placenta to infect fetuses (French et al., 1974; Vantsis et al., 1976). In the first of these reports an Australian agent was recovered readily from lambs born to ewes infected at a range of gestation dates, but the lambs were born apparently normal. In the second report, however, BDV-M was highly pathogenic, and was recovered from fresh aborted fetuses.



The pathogenicity of the two low passage pestivirus reference strains has been proven by the experiments reported here. The low passage BDV-M, which was cultured under conditions which minimised the chances of introducing a contaminating pestivirus, could be considered for use in a superinfection experiment. This experiment is described in the next Chapter.

## CHAPTER 6

### THE RESPONSE OF RECOVERED FIELD CASES OF BORDER DISEASE TO EXPERIMENTAL INFECTION WITH BDV-M

#### INTRODUCTION

The experiments reported in Chapter 4 showed that sheep persistently infected with BD virus, following early infection in-utero, responded dramatically when injected with high passage BDV-M virus. This was a previously unreported phenomenon in sheep. The enteric lesions, however, were very similar to those present in calves dying of mucosal disease. The pathogenesis of MD is still not fully resolved, but in the light of the findings described in Chapter 4 previous reports on the accidental induction of MD were re-examined.

Peter et al. (1967) described a mucosal disease-like syndrome which occurred in a small number of cattle in a few herds, within 10 to 20 days of vaccination with a live attenuated BVD virus vaccine. The pathogenesis of this condition was not understood, and although the vaccine was considered to be aetiologically involved, it was believed that the condition was primarily caused by failure of the immune mechanism in the affected animals. Nearly all the animals which died were seronegative to BVD virus, a regular finding in field cases of MD (see Chapter 1).

Liess et al.(1974) selected 4 seronegative animals from herds in which more than 90% of the cattle were seropositive. Following intra-nasal inoculation with virulent BVD virus two developed an acute disease followed by antibody formation, while two developed a persistent

viraemia. There was, however, no mention of viraemia being looked for before inoculation. One of the latter two animals was killed and showed chronic pneumonia and catarrhal enteritis, but the other died 52 days post-inoculation with typical lesions of MD.

Steck et al.(1980), also examined a number of seronegative cattle. In 56 herds in which 68.8% of animals had neutralising antibodies to BVD virus there were 515 seronegative animals. Two hundred and nine of these were vaccinated with a live attenuated BVD vaccine. Two animals died shortly after vaccination but all the others developed antibody to the vaccine strain. Four such animals later died of MD apparently caused by a BVD virus antigenically distinguishable from the vaccine virus. These results led to the conclusion that BVD-vaccines should include a broader spectrum of antigenic variants. The authors also speculated that animals predisposed to MD or incubating MD virus were among those successfully vaccinated, and they suggested that the parenteral inoculation of a large dose of attenuated live virus would be able to overcome the immune non-responsiveness and thereby confer protection.

In cattle, the evidence from these previous reports was that MD could occur in a small number of animals, which had some immune deficiency, following exposure to BVD virus. The results in sheep reported in Chapter 4 suggested strongly that this immune deficiency, which was a prerequisite for the development of MD, was associated with early intra-uterine exposure to BVDV and the consequent establishment of a persistent infection.

Sheep and cattle persistently infected with pestiviruses are efficient spreaders of virus and play a key role in the epizootiology of BD and BVD (French et al., 1974; Barlow et al., 1980b). An understanding of their response to live pestivirus, both 'wild-type' and vaccine virus, is therefore, crucial to any control policy. In particular, it is vital to know if surviving animals continue to excrete virus, and thus remain a source of infection. In the following experiment two separate groups of recovered field cases of BD and one group of normal lambs were injected with low passage level BDV-M virus.

#### MATERIALS AND METHODS

Animals. Three groups of 5 lambs were used. Field cases were obtained from 2 separate natural outbreaks of BD in Wales. One group of Speckled Beulah lambs (Recovered hairy shaker (RHS) 1) was brought to Moredun when approximately 6 months old. A second group of Suffolk x Speckled Beulah lambs (RHS 2) had been separated from their dams and brought to Moredun earlier when 3 to 4 weeks old. Both groups were kept isolated from each other and from other stock. The isolation of non-cytopathic BD virus from blood and nasal secretions, confirmed that the lambs were persistently infected.

Healthy Cheviot lambs, in whose blood neither BD virus or antibody could be detected, were used as control animals. They had been born and raised at pasture but were housed under similar conditions to the Welsh lambs for 5 weeks before receiving virus.

At the time of challenge the Welsh lambs were approximately 9 months old and the Cheviot lambs were 7-8 months old. Ten weeks after challenge each group was rehoused and at this time two sentinel lambs without neutralising antibody to BDV-M or BVDV-NADL, and without detectable BD virus in their blood, were added to each group. Six weeks later surviving RHS, and two Cheviot lambs from the control group, were killed for pathological and virological examination.

Virus inoculum. BDV-M was grown in secondary FLK cultures from the third tissue culture pass. Each lamb received a 2 ml inoculation of clarified virus suspension containing  $6.1 \log_{10} \text{TCID}_{50}$  per ml given subcutaneously over the left shoulder.

Clinical examination and sampling procedures. Rectal temperatures were taken daily for 4 weeks after virus inoculation and clinical observations were made daily throughout the course of the experiment.

Lambs were bled before and regularly for 10 weeks after infection. Seven ml of blood was collected into evacuated glass tubes containing 70 iu of preservative-free heparin. After thorough mixing 1 ml was removed for haematology and the remainder was centrifuged at  $2,000 \times g$  for 15 minutes. Plasma was collected for antibody determination and buffy coat cells used for virus isolation, as described in Chapter 2.

After mixing with the sentinel lambs, animals were bled at weekly intervals into preservative-free evacuated glass tubes and the serum collected for antibody measurement.

Samples were also collected from any animal showing marked clinical symptoms and these were examined for the presence of microbial pathogens as described in Chapter 2.

Haematological examinations. Full haematological examinations were carried out as described in Chapter 2. To detect significant differences between the groups the results from each bleeding were tested by analysis of variance. The student's t test was used to compare group mean values of each test after infection, with the group mean of two samples taken before infection.

Pathological examinations. All animals dying or killed were examined pathologically, and tissue samples collected for virus isolation as described in Chapter 2.

## RESULTS.

Clinical observations. Control lambs showed the least evidence of disease. Two animals showed biphasic pyrexia ( $>40^{\circ}\text{C}$ ) on days 8 and 11 and the group mean was highest on these days (Fig.6.1). Between 14 and 24 days after infection all animals in this group developed bilateral serous nasal discharges and sporadic coughs; one animal had a mild bilateral conjunctivitis between days 20 and 24.

Animals in the RHS 2 group of persistently infected lambs showed evidence of disease similar to the controls. All had mild pyrexia on some days between 9 and 12 days after infection with the group mean being highest on day 9. (Fig.6.1). Sporadic coughing and serous nasal discharge were apparent in all animals between 18 and 24 days after infection. In one animal (532) the nasal discharge became progressively worse over the next ten weeks. There was a

gradual change from a serous to a mucopurulent discharge, which became encrusted around the nares and over the muzzle with excoriation of the underlying epithelium. The animal was killed on humanitarian grounds 92 days after infection.

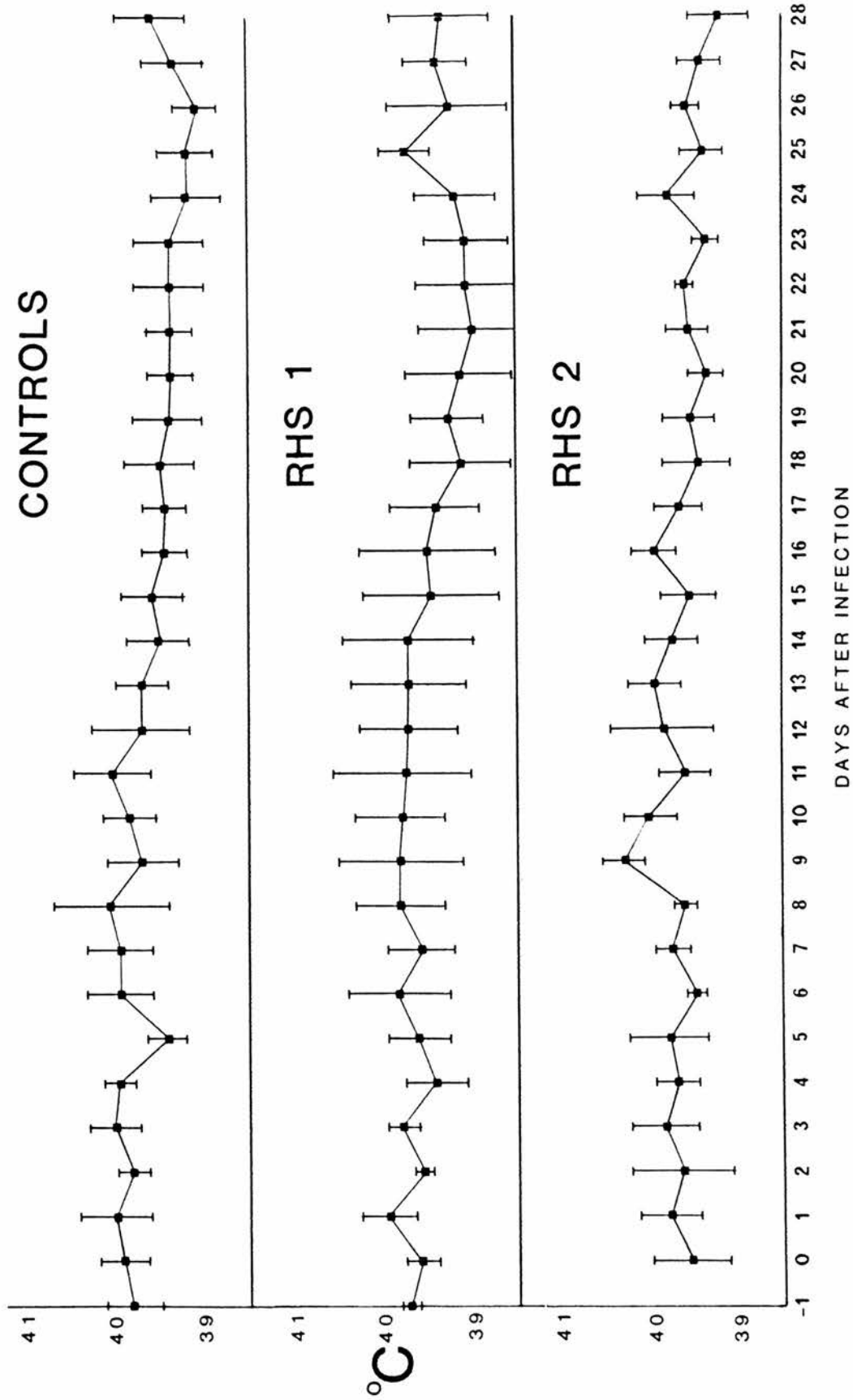
The most severe disease was seen in the RHS 1 group of persistently infected lambs. In addition to respiratory disease similar to that seen in the other groups, three lambs showed depression, anorexia and diarrhoea at varying times after infection. One animal (1247) had pyrexia ( $40.0^{\circ}\text{C}$  to  $40.8^{\circ}\text{C}$ ) from day 6 to day 16 when it died, and this increased the standard deviation of the group mean (Fig. 6.1). This lamb was depressed from day 7 onwards and started to scour on day 11. Faeces were loose and mucoid, becoming very watery and foul-smelling by day 15. A marked serous oculo-nasal discharge was apparent from day 13 to death. Rehydration therapy with Ion-Aid (Syntex Pharmaceuticals Ltd.) on days 15 and 16 after infection did not prevent death.

A second animal (1246) started to scour 28 days after infection. The scour was intermittent during the next 12 days, but by day 40 it was watery and foul-smelling. The lamb became weak, depressed and anorexic and was killed in-extremis on day 44. This animal had mild pyrexia ( $40.2^{\circ}\text{C}$  to  $40.4^{\circ}\text{C}$ ) on days 5, 8, 10, 11, and 42 so that pyrexia was not a major feature of this lamb's terminal illness.

The third lamb to scour was first seen to do so 101 days after infection. At this time it had a serous nasal discharge and a temperature of  $39.5^{\circ}\text{C}$ . The scour



E 6.1 Rectal temperatures of three groups of lambs  
infected with BDV-M. Mean values  $\pm$  SD.



worsened, and the lamb became weak and anorexic shortly before dying 5 days later.

No disease was observed in the six sentinel lambs during their 6 weeks' co-habitation with the injected groups.

#### Microbiological Findings.

##### Virology.

Live animals. Virus recoveries from the blood of the three groups of lambs are summarised in Figure 6.2. Virus was isolated from all the animals in the control group 6 days after infection. One lamb (402) also yielded virus on days 4, 8 and 11, and virus was recovered from one other lamb 11 days after infection. None of the isolates caused cytopathic changes during two weekly passages in FLK cells.

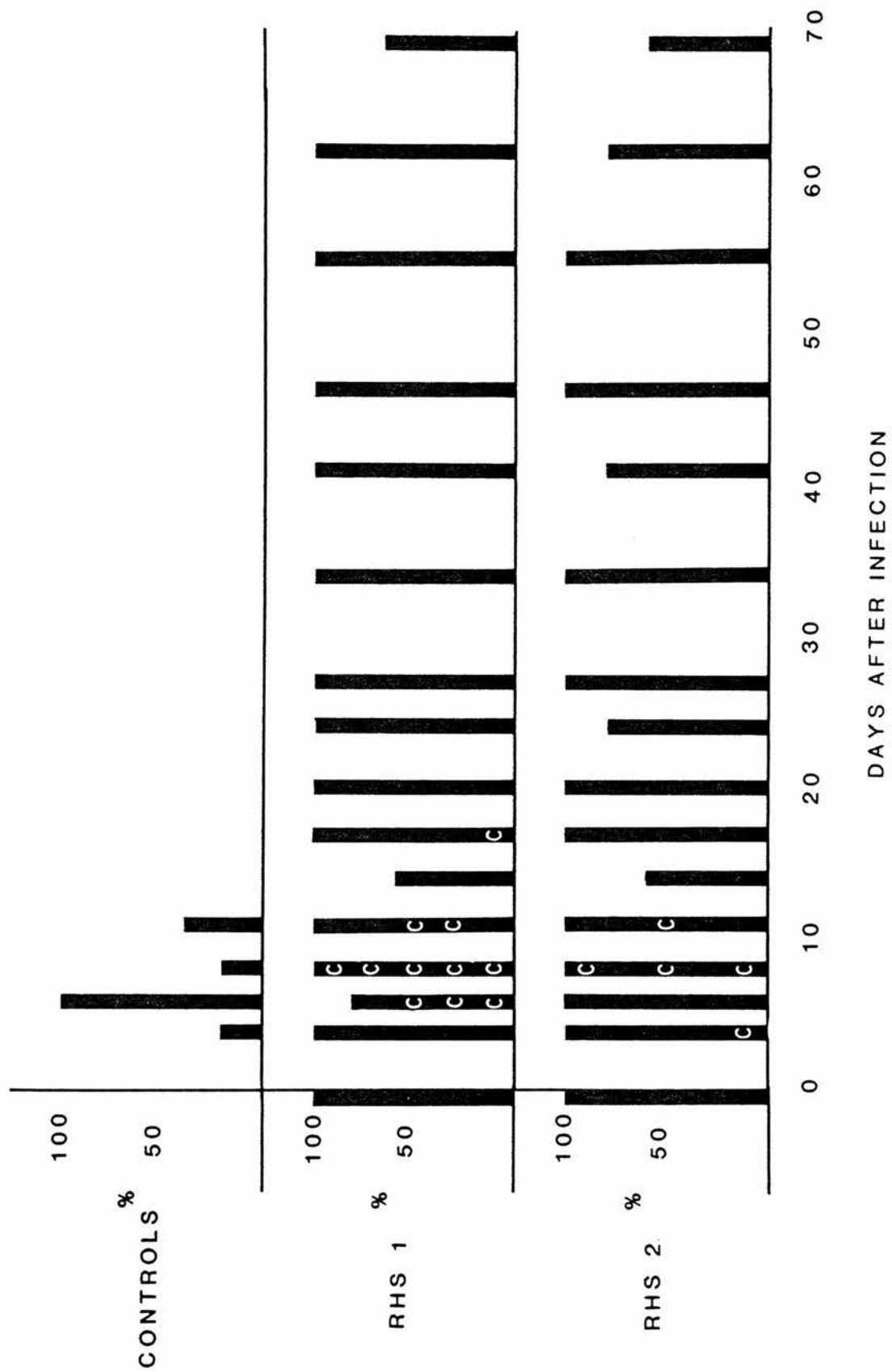
Border disease virus was recovered consistently from the blood of persistently infected lambs before, and throughout the ten weeks after, they received cytopathic BDV-M virus. The majority of isolates were non-cytopathic, but cytopathic effects were seen after 2 weekly passages in cultures exposed to blood from all animals in the RHS 1 group and from 3 of the lambs in the RHS 2 group. Cytopathic virus was recovered only between days 4 and 18 with the majority of isolates being made 8 days after inoculation. All cytopathic isolates, with the exception of the single isolate on day 18, were passaged 6 times and remained cytopathic at each pass.

All samples collected on days 8 and 11 were also passaged 6 times. The classification of isolates as CP or NCP remained the same after the further passages.

FIGURE 6.2      Isolations of BD virus from the blood of three groups of lambs expressed as the % of lambs in each group from which virus was recovered.

- Non-cytopathic BD virus
- C Cytopathic BD virus

Cytopathic virus was isolated from all 5 animals in the RHS1 group and 3 of the 5 animals in the RHS2 group, on the days shown.



Dead animals. Virus recoveries from the four RHS lambs that died or were killed before the end of the experiment are summarised in Table 6.1. Border disease virus was isolated from all samples tested. Cytopathic virus was widespread in the 3 animals in the RHS 1 group, whereas cytopathic virus was recovered only from the mesenteric lymph node of lamb 532 in the RHS 2 group.

Virus recoveries from the lambs killed at the end of the experiment are summarised in Table 6.2. No virus was isolated from the 2 control lambs, but BD virus was recovered from all the samples cultured from the RHS lambs. Cytopathic virus was recovered only from 3 samples from one of the two survivors in the RHS 1 group.

#### Other Microbiological Findings

Ocular swabs from the lamb in the control group with conjunctivitis yielded Neisseria ovis and Mycoplasma conjunctivae.

No pathogenic bacteria were grown from rectal swabs taken from the 3 lambs that scoured.

Pasteurella haemolytica Type T4 and an untyped Streptococcus were isolated from the retropharyngeal lymph node of the lamb with nasal ulceration.

#### Haematology

There were differences in the haematological responses of the 3 groups to infection with BDV-M. This was most apparent with the total and differential leucocyte counts. The control and RHS 1 groups, but not the RHS 2 group, showed evidence of reduced total leucocyte counts (Figure 6.3). In the control group the reduction was

TABLE 6.1

Border disease virus recoveries from RHS lambs dying  
or killed on humanitarian grounds

Group Number	RHS 1			RHS2
Lamb Number	1247	1246	1249	532
Day of Death	16	44	106	92
Died/Killed	Died	Killed	Died	Killed
Samples Tested				
Blood clot	CP	CP	CP	NCP
Faeces	NT	CP	NCP	NT
Spleen	CP	CP	CP	NCP
Mesenteric lymph node	CP	CP	CP	CP
Colon	CP	CP	CP	NCP
Ovary/Testis	CP	CP	NCP	NCP
Cerebellum	CP	NCP	CP	NCP
Kidney	CP	NCP	CP	NT
Lung	CP	CP	NT	NCP
Abomasum	NT	CP	CP	NT
Bone Marrow	CP	NT	CP	NCP
Nasal ulcer	NT	NT	NT	NCP

CP, Cytopathic virus

NCP, Non-cytopathic virus

NT, Not tested



TABLE 6.2

Virus recoveries from lambs sacrificed  
at the end of the experiment.

Samples Tested	Group/Lamb number							
	Control		RHS 1		RHS 2			
	402	412	1245	1248	528	531	533	535
Buffy coat	-	-	NCP	CP	NCP	NCP	NCP	NCP
Spleen	-	-	NCP	NCP	NCP	NCP	NCP	NCP
Colon plus mesenteric lymph node	-	-	NCP	CP	NCP	NCP	NCP	NCP
Bone Marrow	-	-	NCP	NCP	NCP	NCP	NCP	NCP
Testis/ ovary	NT	NT	NCP	CP	NCP	NCP	NCP	NCP
Cerebellum	-	-	NCP	NCP	NCP	NCP	NCP	NCP

-, No virus isolated

NT, Not tested, lambs were castrates.

NCP, Non-cytopathic virus. CP, Cytopathic virus

significant ( $p < 0.05$ ) on day 21; the RHS 1 group showed the same degree of significant reduction on days 4 and 14. The increase in the leucocyte count of the RHS 2 group was significant ( $p < 0.05$ ) on days 18 and 35 after infection. Significant differences between the responses of the groups were detected by analysis of variance as shown on Fig. 6.3; These were most marked 14, 18, 25 and 63 days after infection and in all instances were due to low counts in the RHS 1 group compared to the other two groups.

Differential leucocyte counts revealed evidence of variable degrees of lymphocytopaenia and neutrophilia in all groups. There were no obvious differences in monocyte, eosinophil or basophil counts in any of the groups following infection with BDV-M.

Lymphocytopaenia was most marked in the group (RHS 1) with the worst disease (Figure 6.4). Of the two lambs dying during the period of haematological examination, both suffered severe lymphocytopaenia. Lamb number 1247 had lymphocyte counts before infection of 6043(79%) and 3656(73.5%)  $\times 10^9/l$ . The counts obtained 4, 6, 8, 11, and 14 days after infection were 4143(80%), 2145(41.5%), 907(35%), 2069(36%) and 451(23%) respectively. At all times after infection, the lamb killed 44 days after infection (No. 1246) had reduced counts which went from 7013(89%) and 4807(84.5%) before infection to 2362(22.5%) two days before death. Lymphocytopaenia in the control group occurred most noticeably on days 4, 11, 14 and 21 after infection but these reductions were not significantly different from the mean value of the group

FIGURE 6.3      Mean total leucocyte counts of the 3 groups of lambs.

●—● Controls      ▲—▲ RHS1      ■—■ RHS2

Levels of significance of differences between the groups as revealed by analysis of variance are shown by the asterisks.

- \*       $p < 0.05 > 0.01$
- \*\*      $p < 0.01 > 0.001$
- \*\*\*    $p < 0.001$

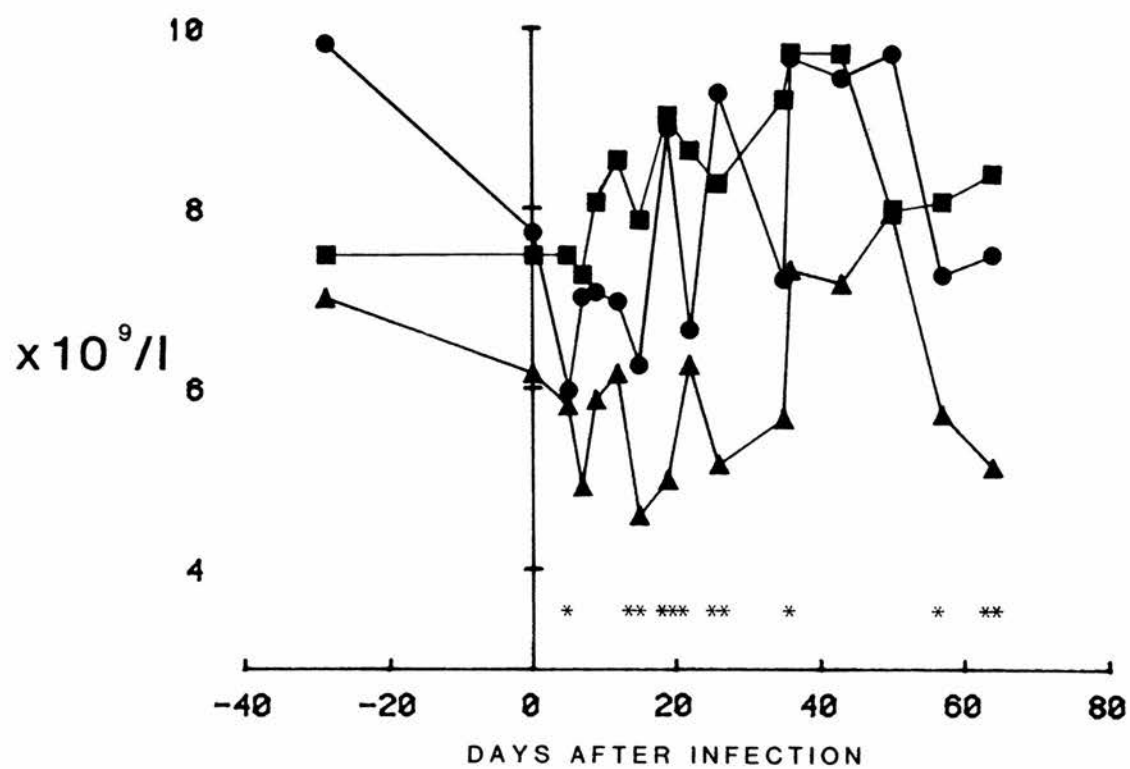
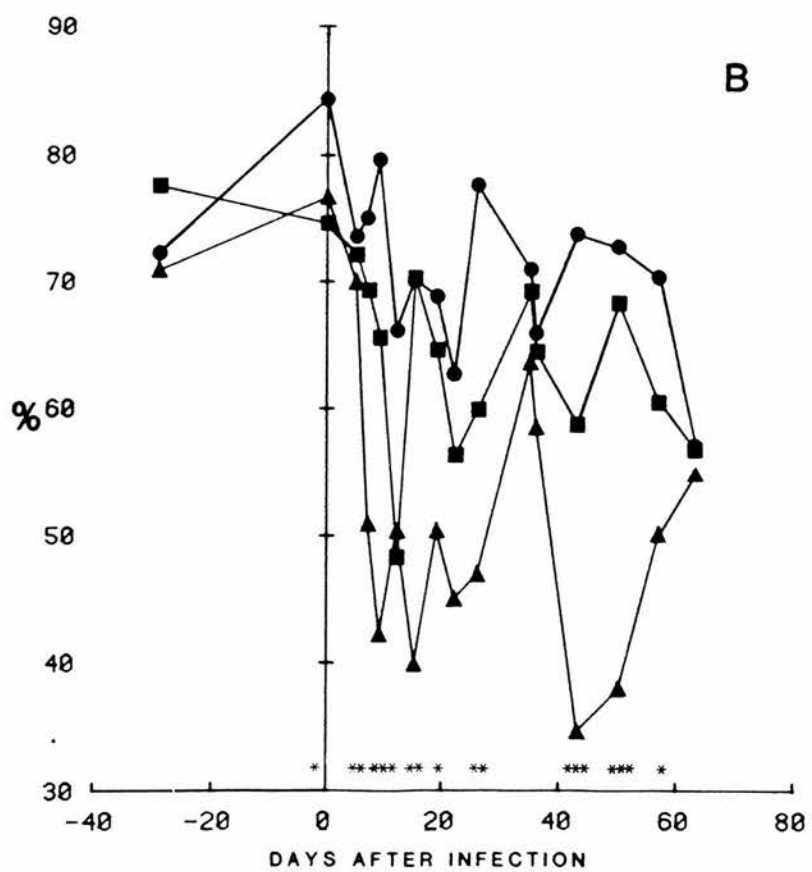
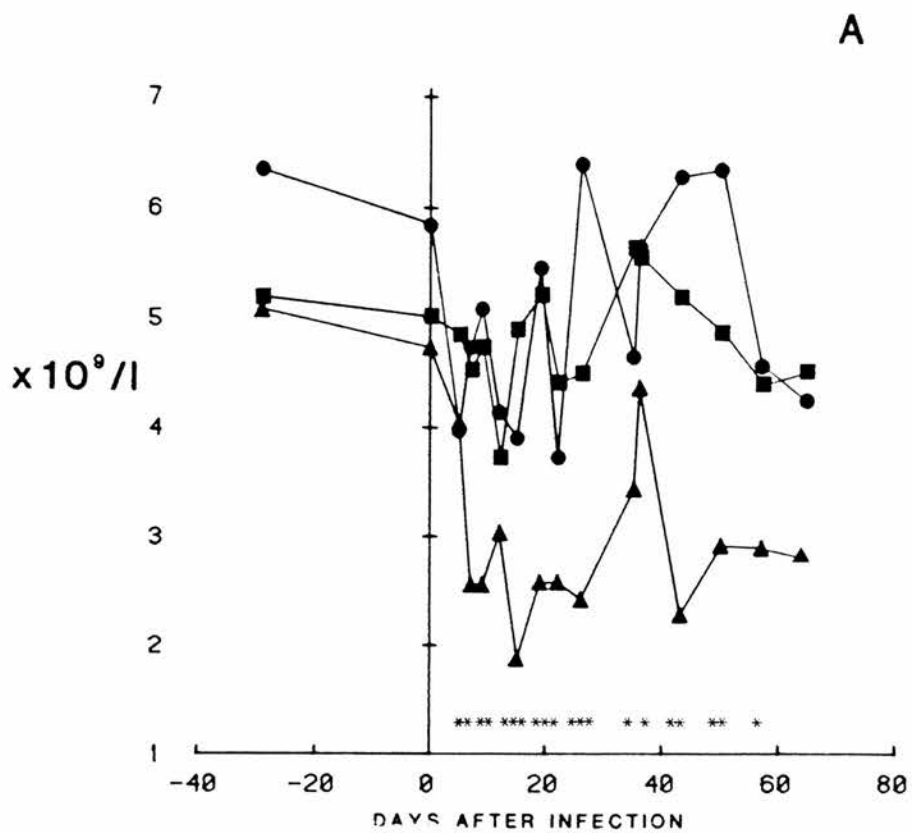


FIGURE 6.4      Mean mononuclear leucocyte cell counts of the 3 groups of lambs. A. Absolute values B. Percentage of total leucocyte counts.

●—● Controls      ▲—▲ RHS1      ■—■ RHS2

Levels of significance of differences between the groups as revealed by analysis of variance are shown by the asterisks.

\*       $p < 0.05 > 0.01$   
 \*\*      $p < 0.01 > 0.001$   
 \*\*\*     $p < 0.001$



before infection. Similarly, lymphocytopenia in the RHS 2 group, was most apparent 11 days after infection but did not differ significantly from the mean value before infection.

Neutrophilia was seen in all 3 groups. Variable responses between groups were significant 8, 11, 42 and 49 days after infection and resulted from differences between the control group and one or other of the RHS groups (Figure 6.5).

The mean polymorphonuclear leucocyte counts of the control group and the RHS 1 group were not significantly higher than before infection on any day up until 35 days after infection. On day 11 the mean of the RHS 2 group was significantly higher ( $p < 0.01$ ) than before infection.

Haematological values relating to erythrocytes are summarised in Figure 6.6. Before infection, the control group had significantly higher haemoglobin and PCV values than the two RHS groups. This continued after infection and accounted for the significant differences between the groups recorded for these values and the mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC). Significant differences between the groups' erythrocyte counts were not consistent. Twice the main variation was between the RHS groups and on 2 other occasions the difference was between the controls and the RHS 2 group.

All groups showed reduced erythrocyte counts following infection. In the control group this was significant ( $p < 0.05$ ) from day 14 to day 28. In the RHS 1



FIGURE 6.5 Mean polymorphonuclear leucocyte cell counts of the 3 groups of lambs. A. Absolute values B. Percentage of total leucocyte count.

●—● Controls      ▲—▲ RHS1      ■—■ RHS2

Levels of significance of differences between the groups as revealed by analysis of variance are shown by the asterisks.

- \*     $p < 0.05 > 0.01$
- \*\*    $p < 0.01 > 0.001$
- \*\*\*  $p < 0.001$

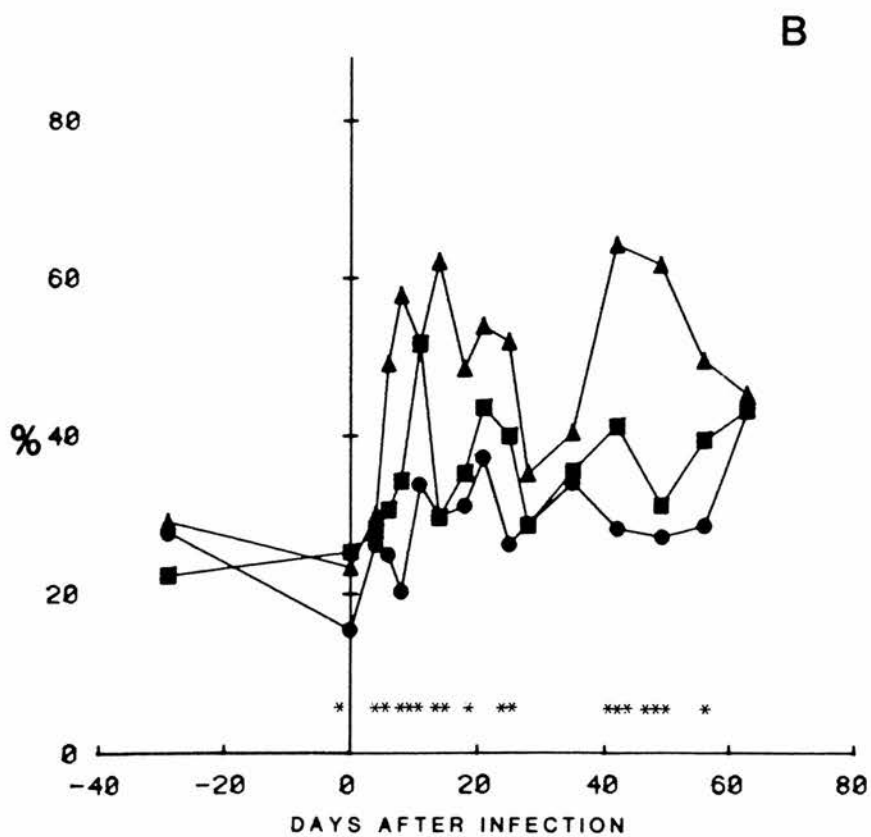
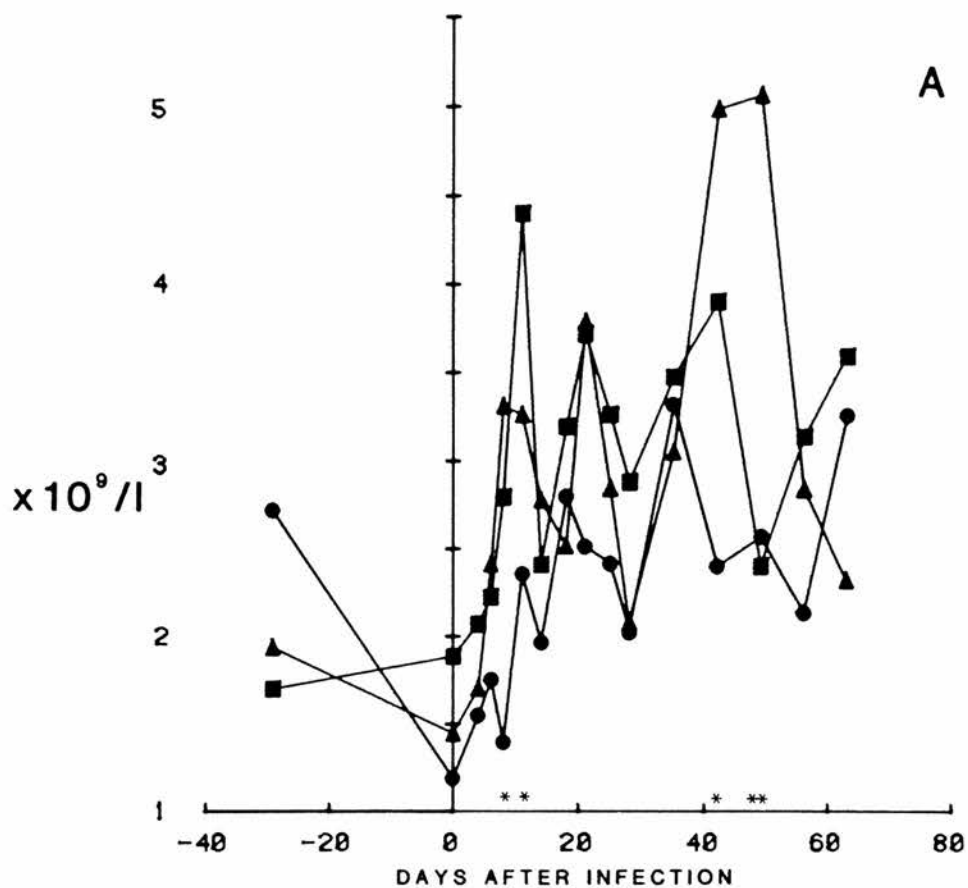


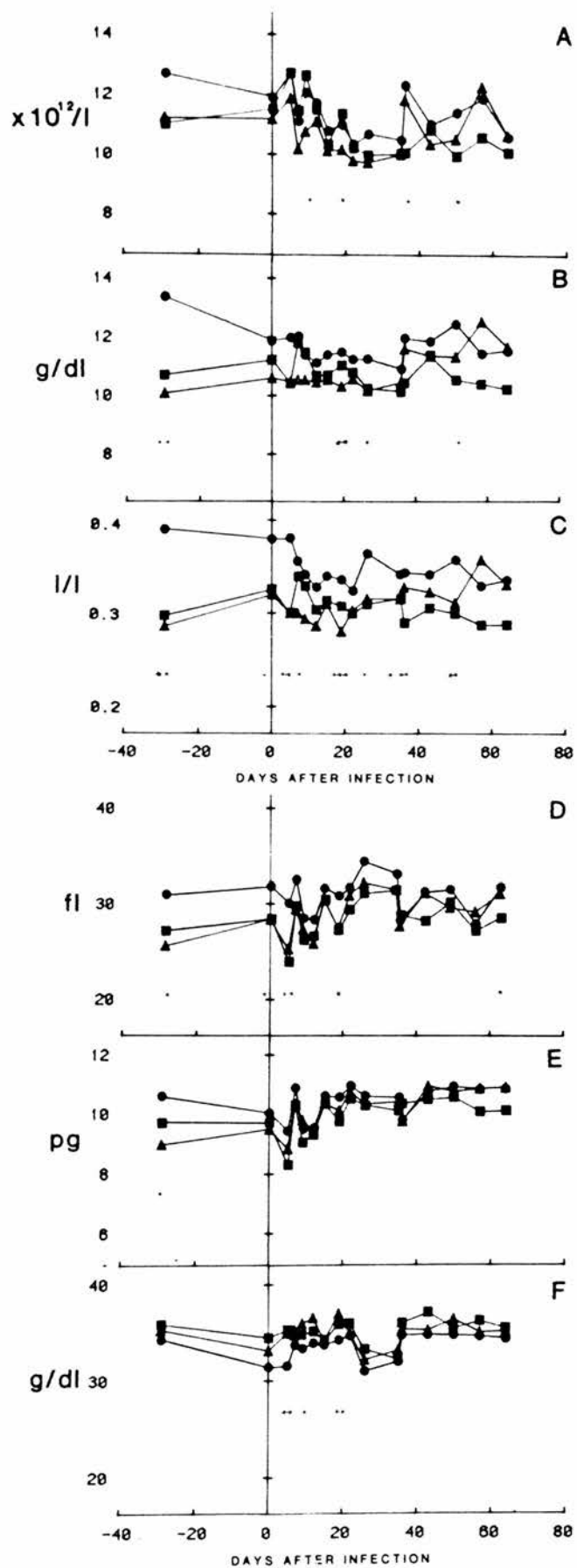
FIGURE 6.6      Group mean haematological values relating to erythrocytes.

- A. Erythrocyte counts
- B. Haemoglobin
- C. Packed cell volume (PCV)
- D. Mean corpuscular volume (MCV)
- E. Mean corpuscular haemoglobin (MCH)
- F. Mean corpuscular haemoglobin concentration (MCHC)

●—● Controls      ▲—▲ RHS1      ■—■ RHS2

Levels of significance of differences between the groups as revealed by analysis of variance are shown by the asterisks.

\*       $p < 0.05 > 0.01$   
\*\*       $p < 0.01 > 0.001$   
\*\*\*       $p < 0.001$



group a similar reduction was only apparent on day 14. The degree of reduction in the RHS 2 group was the same on days 14 and 21, but more significant on days 25 ( $p < 0.01$ ) and 28 and 35 ( $p < 0.001$ ). Haemoglobin levels declined significantly only in the control group on days 8 and 14 and from day 21 to 28. The same was true for the PCV when significant reductions were detected on days 6 to 11, 18 to 21 and 28 to 35.

### Serology

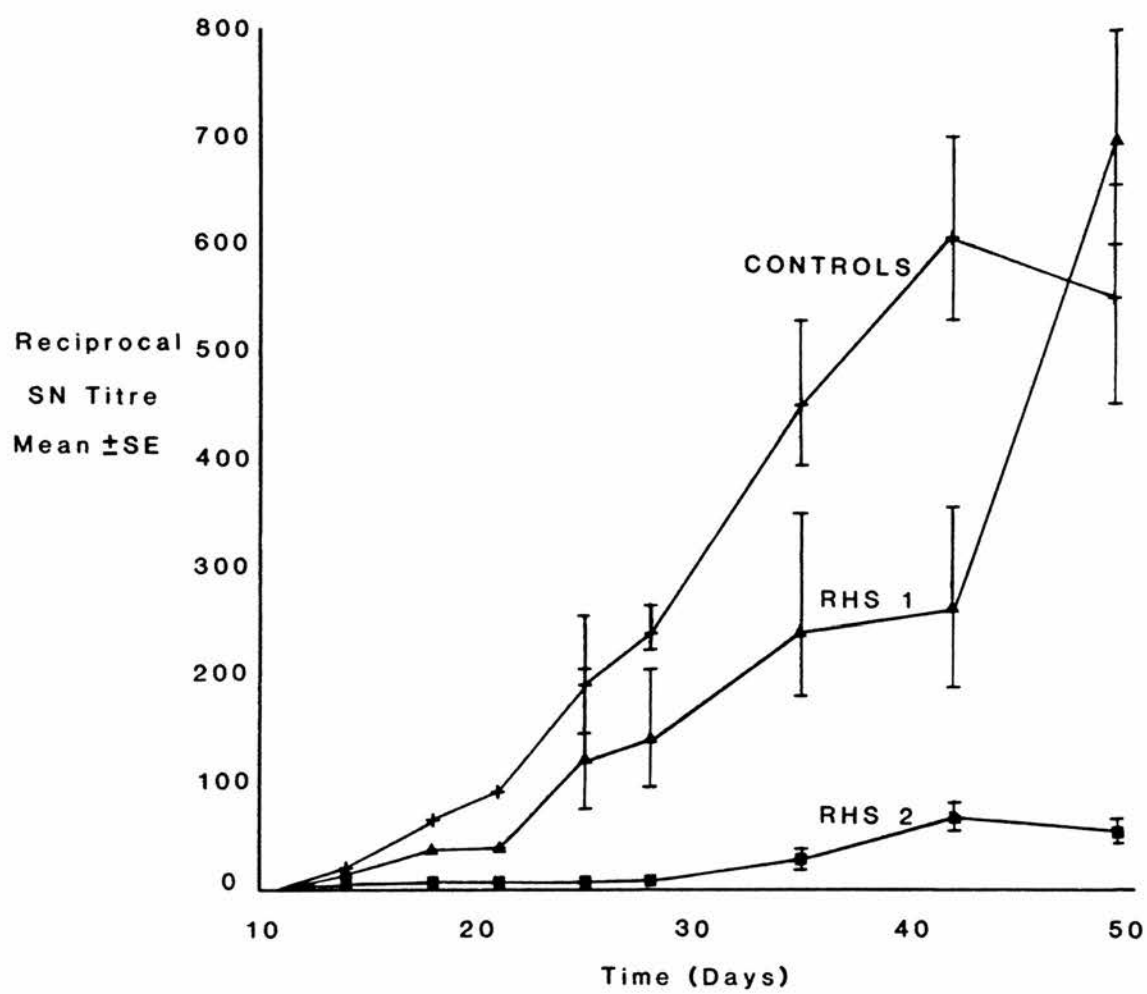
#### Three groups following injection with BDV-M virus

Neutralising antibody to BDV-M was first detected in the plasma of 3 animals 11 days after infection. On day 14 only 3 animals, all in RHS 2, had not seroconverted, and by day 18 all lambs were seropositive. The lamb that died 16 days after infection (No.1247 from RHS 1 group) had a titre of 11 two days before death.

The rate of development of neutralising antibodies by the two groups of RHS was markedly different (Figure 6.7). The group in which lambs scoured and died (RHS 1) developed antibodies at the same rate as the controls. Only on day 42, shortly before the death of lamb 1246, which had the lowest titre in the group was there a significant difference ( $p < 0.05$ ) between the group mean and that of the controls. From day 49 to the end of the experiment, the mean titre of the survivors of the RHS 1 group was consistently higher than that of the controls.

The RHS 2 group seroconverted very slowly to the virus; the difference between the group mean and that of the controls was highly significant ( $p < 0.001$ ) from day 21

FIGURE 6.7      The development of specific neutralising antibody to BDV-M by the three groups of lambs. The results are shown as geometric means with the bar lines representing the standard error of the group mean.





to day 49. During the next two weeks the group geometric mean rose to 222 and stayed around this level for the remainder of the experiment.

Only the control group produced cross-neutralising antibody to BVDV-NADL virus. Three lambs had antibody 18 days after infection and all were seropositive by day 25. There was considerable individual variation, but the group mean rose slowly reaching a plateau 56 days after infection. The mean neutralising antibody titres of all three groups to BDV-M and BVDV-NADL ten weeks after injection with BDV-M are shown in Table 6.3.

#### Sentinel lambs after mixing with the three groups

Sentinel lambs mixed with the RHS 1 and RHS 2 groups seroconverted to BDV-M and BVDV-NADL viruses, whereas the sentinel lambs mixed with the control group remained seronegative to both viruses. The serum neutralising antibody titres of the sentinel lambs 3 weeks and 6 weeks after mixing with their respective groups are shown in Table 6.4.

#### PATHOLOGY

Lambs dying or killed on humanitarian grounds. All four lambs had residual CNS lesions consistent with an in-utero infection with BD virus.

Of the three lambs from the RHS 1 group that developed diarrhoea, the first to die 16 days after infection had an atrophic thymus and obvious enteric lesions. This lamb had gross thickening of the caecum, colon and ileum, and the mesenteric lymph nodes were enlarged. Histologically there was hyperplastic

TABLE 6.3

Geometric mean (range) neutralising antibody titre  
of the 3 groups of lambs 10 weeks after being  
injected with BDV-M

GROUP	ANTIBODY AGAINST	
	BDV-M	BVDV-NADL
CONTROLS n = 5	510 (256 - 720)	84 (45 - 256)
RHS 1 n = 3	1625 (1024 - 2048)	<4
RHS 2 n = 5	255 (64 - 720)	<4

TABLE 6.4

Neutralising antibody titres of sentinel lambs after  
mixing with the 3 groups of lambs injected with BD-M  
10 weeks previously

Sentinels mixed with	Days after mixing	Neutralising antibody to BDV-M	BVDV-NADL
CONTROLS	0	<4 +	<4
	21	<4	<4
	42	<4	<4
RHS 1	0	<4	<4
	21	45 , 20 *	<4
	42	202 , 101	16 , 4
RHS 2	0	<4	<4
	21	80 , 6	<4
	42	227 , 321	9 , 90

+ Both sentinel lambs <4.

\* The values for each sentinel lamb are given.

enteropathy with glandular invasion of the submucosa, without obvious inflammation. Gross enteric lesions were not seen in the other two lambs, but the lamb killed 44 days after infection had histological evidence of mucosal hyperplasia, again without obvious inflammation. Death of the third lamb 106 days after infection was attributable to pulmonary congestion and oedema with endobronchial haemorrhage; the cause was obscure but could have been associated with the administration of oral rehydration therapy.

The lamb from the RHS 2 group killed 92 after infection had foul necrotic ulceration around the nares and lips. Histologically, the lip lesion showed non-specific ulceration, haemorrhage with a mixed inflammatory infiltrate and acanthosis. The retropharyngeal lymph nodes were enlarged, but there was no evidence of disease elsewhere in the respiratory tract.

Lambs killed 16 weeks after infection with BDV-M virus. No gross or histological abnormalities were recognised in the two control lambs. Compared with the RHS lambs in the experiment, splenic follicles and Peyer's patches appeared better developed; in the thymus, the cortices were thicker and better differentiated and the Hassal's corpuscles were in a more advanced state of development.

The six surviving RHS lambs all had residual CNS lesions of in-utero BD virus infection. The two lambs from the RHS 1 group both had mild mucosal hyperplasia and lymphoid infiltration of the lamina propria and submucosa of the ileum and caecum. One lamb had a mild lymphoid

infiltration of the renal pelvis, and the other had a slight focal choroiditis.

No consistent lesions were present in the 4 survivors of the RHS 2 group. Mild focal areas of non suppurative meningitis, encephalitis, myocarditis, pancreatitis, and nephritis were recorded in one or more of the lambs.

### DISCUSSION

Each of the 3 groups of lambs responded differently to infection with low passage BDV-M.

The normal control animals showed evidence of transient pyrexia and mild disease 1 to 3 weeks after infection. These findings, together with a short-lived viraemia and rapid development of specific neutralising antibody were consistent with the results of previous experimental infections of normal sheep (Shaw et al., 1967; Roeder et al., 1983; Terlecki and Roeder, 1983).

The differing responses of the 2 groups of RHS lambs to BDV-M frustrate a single explanation of the pathogenesis of such an infection. Nevertheless, the finding that such animals remain effective excretors of virus even though they have responded serologically to a superinfecting pestivirus has serious practical consequences for the control of pestivirus-induced disease by vaccination. Recent analogous findings in cattle have been reported. Liess et al. (1983) vaccinated 5 persistently viraemic cattle with a live attenuated, cytopathic BVDV vaccine. They all seroconverted to the vaccine virus strain and at least one other reference

strain, but not to a cytopathic field isolate from the herd of origin of one of the 5 cattle. Four of the 5 animals developed a slowly progressing "runt-disease" like state, and succumbed or had to be sacrificed 21 to 93 weeks after vaccination. Persistent viraemia with non-cytopathic BVDV was demonstrated regularly in the 4 animals until death. Virus isolation was not attempted from dead animals, but BVDV was demonstrated in a wide range of tissues by IFT. Ernst and Butler (1983), without detailed virological studies, have also provided good evidence that cattle can die of BVDV-associated disease in spite of seroconverting well to a live BVDV vaccine.

In this experiment further evidence was provided of the specificity of the neutralising antibody response of RHS to pestivirus infections. No cross-neutralising antibody to BVDV-NADL was produced, in contrast to the control group and the seroconverting sentinel lambs. The difference in the rate of development of the serological response to BDV-M by the two groups of RHS lambs was highly significant. The rapid development of neutralising antibodies in the group (RHS 1) that suffered fatalities and the slow rise of such antibodies in the group (RHS 2) that was comparatively disease-free suggest that humoral immune mechanisms are not essential for the control of BD virus replication in such animals.

The significant lymphocytopaenia in the RHS 1 group, particularly the dying animals, suggest that lymphocyte depletion or mobilisation to tissue sites is important in the pathogenesis of disease in superinfected persistently

infected animals. No other studies in comparable sheep have yet been reported, but Roeder (1984) has recently shown that lymphocytopaenia is a common sequel to experimental infection of normal healthy lambs. Findings in the control group in this experiment would support this. Vantsis et al., (1979), however, have demonstrated that neutropaenia can also occur following the experimental infection of normal sheep with BD virus (Vantsis et al., 1979). Haematological data from BVDV infections in cattle are also conflicting. Whilst leucopaenia has been widely recorded this has been attributed variously to decreases in the numbers of neutrophils, lymphocytes or both; neutrophilia in animals dying of chronic disease has also been reported. (Pritchard, 1963). A recent study in normal cattle infected with BVDV has recorded a decrease in the absolute numbers of B and T lymphocytes and in the percentage of T lymphocytes (Bolin et al., 1985a). The same group of workers has also shown that persistently infected cattle superinfected with cytopathic BVDV suffer a significant decrease of T lymphocytes (Bolin et al., 1985b).

The BDV-M used for infection was cytopathic in tissue culture. Virus recovered from the blood of the control lambs was non-cytopathic. It has been shown in studies of the recovery of BVDV-NADL from experimentally infected sheep that virus could be detected by IFT one passage before cytopathic effects were seen, and that 3 passages were required before CPE was obvious (Hassan, 1979). In spite of up to 6 passages, virus from the control lambs in this experiment remained

non-cytopathic. The isolation of cytopathic virus from the blood of the RHS groups occurred over a short time scale, but apparent persistence of cytopathic virus in tissues of the RHS 1 group was associated with disease in this group. Recently reported findings in cattle, in which distinct aetiological roles have been assigned to non-cytopathic and cytopathic BVD viruses suggest that the balance of the two populations is important for the precipitation of clinical disease (Brownlie et al., 1984; Bolin et al., 1985b).



CHAPTER 7PLAQUE PURIFICATION AND CHARACTERISATION OF TWO  
REFERENCE STRAINS AND THREE FIELD ISOLATES OF  
CYTOPATHIC PESTIVIRUSESINTRODUCTION

The results of the neutralisation tests described in Chapter 5 confirmed the antigenic relationship between BVDV-NADL and BDV-M established by previous workers (Vantsis et al., 1976; Wrathall et al., 1978; Laude and Gelfi, 1979; Hassan, 1979). The degree and nature of this serological relationship between the two viruses remains largely unexplored, as does their relationship with field isolates of pestivirus. In this Chapter, results are presented of some comparative aspects of the growth, physical properties and pathogenicities of plaque purified stocks of the two reference viruses and three cytopathic BVDV field isolates.

MATERIALS AND METHODS

Cell cultures. Semi-continuous cell lines were prepared by trypsin disaggregation of foetal lamb brains (FLB). They were checked regularly for the presence of contaminating NCP pestivirus by IF tests, and were used between passage levels 5 and 50. Secondary FLK cells and BEK cells used for virus isolation were prepared as described previously, and all cells were grown in supplemented Eagle's MEM (Chapter 2).

Viruses. The low passage virus stock of BDV-M, derived from the IIB brain pool, was passaged a further 4 times in

secondary FLK cells, and a stock prepared at passage level 7, to match the passage level of the BVDV-NADL stock. Three cytopathic BVD viruses isolated from cases of MD in different regions of Scotland (Table 2.1) were available at the 2nd passage level in BEK cells. Virus had been isolated from different samples from each animal; a nasal swab (BVDV-7446), a spleen/mesenteric lymph node pool (BVDV-8085) and a retropharyngeal lymph node (BVDV-B816).

All viruses were plaque purified 3 times in FLB cells. Master stocks of each virus were grown, and working stocks prepared from these as required. At every stage of these procedures viruses were grown in the same batch of cells, and uninfected cells were passaged along with the viruses.

Plaque assay. The procedure was based on that described by Laude and Gelfi (1979). Suspensions of FLB cells at a concentration of  $2 \times 10^5$  per ml were added to 55 mm diameter plastic petri dishes (Sterilin Ltd.) or 12 well Linbro plastic plates (Flow Laboratories Ltd.). The petri dishes received 8 ml of suspension and each well of the Linbro plates 1.5 ml of suspension. Cells were incubated at 37°C in a humidified atmosphere containing 5 per cent CO<sub>2</sub>, for 24 to 48 hours, until confluent monolayers were formed. Growth medium was then removed and the cells washed three times with Hanks' BSS.

Serial dilutions of virus, prepared in bijou bottles were added in 0.2 ml volumes to duplicate cell monolayers, and allowed to adsorb at 37°C for two hours. After this time, petri dishes received 8 ml and Linbro plate wells

received 2 ml of overlay medium. The overlay consisted of '199' medium supplemented with BSA (0.5%), LAH (0.1%), yeast extract (0.1%), and heat-inactivated horse serum (3%), and contained 100 iu/ml penicillin, 100 µg/ml streptomycin, 2 µg/ml amphotericin B deoxycholate and 0.6% agarose (Sea-plaque, FMC Corporation, Rockland, USA).

Plates were left on the bench until the overlay medium had solidified, and were then returned to the gassed incubator. Four to five days later, cells were fixed with 10% formol saline and stained with 1% (w/v) crystal violet in 20% (v/v) ethanol in distilled water.

#### Virus characterisation.

Immunofluorescence and neutralisation tests. Stocks of the five viruses and the uninfected control cells were tested for specific fluorescence by inoculation of 0.2 ml volumes into each of four tubes containing coverslips. In two tubes FLK cells were growing, while the other two contained BEK cells. After 72 hours' incubation the coverslips were fixed and stained in IIF tests as described in Chapter 2.

Ten-fold virus dilutions were tested against 1:20 dilutions of convalescent calf serum (385) and lamb serum (D1479/14) in a microneutralisation assay.

Growth characteristics. The development of CPE caused by the 5 viruses was studied by inoculating, at a m.o.i. of 1, FLB cells grown on coverslips, which were fixed and stained with May-Grunwald Giemsa firstly at six-hour and then at 12-hour intervals. Coverslips infected the same way were also fixed and stained at the same time intervals in IIF tests as described in Chapter 2.

Growth curves of 3 of the viruses in FLB cells were studied using confluent monolayers of cells grown in 15 cm x 1.5 cm glass test tubes. At the start of the experiment the cells in 3 tubes were trypsinised and counted. The mean number of cells was used to adjust the concentration of virus in the inocula so that each tube was infected at a m.o.i. of 1. Growth medium was removed from the cells, which were washed 3 times with Hanks' BSS. Each virus was used to infect 16 tubes. After 2 hours' adsorption at 37°C the inocula were removed, the cells washed twice with '199' maintenance medium (m/m) supplemented with 3% heat-inactivated horse serum, 1 ml of the same medium being added finally to each tube.

At six hour intervals for the first 24 hours, and at 12 hour intervals for the next 144 hours, one tube for each virus was removed from the incubator. Supernatant fluid was aspirated into a vial, and 1 ml of m/m was added to the tube. Vials and tubes were then frozen at -70°C awaiting assay of extracellular and intracellular virus. All samples were assayed in one test 7 weeks later.

Electron microscopy. Negative staining of infected and uninfected disrupted cells, and thin sections of cell pellets were both carried out as described in Chapter 2.

Ether and chloroform sensitivity. Virus suspensions were treated with 50 per cent ether or 20 per cent chloroform at room temperature for 30 minutes, the mixtures being kept agitated constantly. The aqueous phases were removed for virus assay and residual ether was blown off using sterile air. A bovine enterovirus (G1984) was treated the same way and assayed in tube cultures of secondary BEK cells.

Filtration. Sterile 25 mm millipore filters in Swinnex holders (Millipore (UK) Ltd.) were attached to disposable plastic syringes and flushed with Hanks' BSS containing 0.5% BSA. A 10 ml stock of each virus was prepared by filtration through a 200 nm average pore diameter (a.p.d.) filter. Two ml volumes of these stocks were subsequently passed through filters of 100 nm, 50 nm and 25nm a.p.d. All suspensions were kept at 4°C and assayed for virus content the same day.

Pathogenicity for sheep.

Infection of gnotobiotic lambs. Ten hysterectomy-derived, colostrum-deprived gnotobiotic lambs, obtained according to the method of Hart et al.(1971), were reared singly in plastic isolators. In the first experiment, each of five lambs were infected with one or other of the five viruses. One-day-old lambs were infected intranasally with  $3 \times 10^4$  to  $12 \times 10^4$  pfu in a 5 ml dose divided equally between each nostril. Nine days later, lambs received  $10^5$  pfu of virus given intravenously, and they were killed at three weeks of age for the collection of convalescent sera.

The second experiment was similar to the first, except that lambs were injected intravenously at 7 days old, and intramuscularly at 21 days of age with 2 ml of a four-fold concentrate of virus emulsified with an equal volume of Marcol 52/Montanide 888 (Esso Ltd., London /Seppic Ltd., Paris). These inocula were kindly prepared by Miss I. Pow, Moredun Research Institute.

The lambs were exsanguinated under halothane anaesthesia at 6 weeks old and the sera collected for serological comparisons (Chapter 8).

All lambs were bled before, and regularly after infection, for virological and serological testing as described in Chapter 2. Lamb temperatures were recorded daily.

Infection of pregnant ewes. Nineteen oestrus-synchronised ewes were divided into 6 groups. At 52 days' gestation, groups of 3 ewes received one or other of the 5 viruses, and a group of 4 received equivalent passage level tissue culture fluid in a 2 ml dose given subcutaneously. Virus titres were between 5.1 and 5.7 pfu per ml. At 82 days' gestation, the ewes were killed and placenta and foetuses collected for pathological, virological and serological examinations as previously described (Chapter 5 Section 3).

## RESULTS

Susceptibility of FLB cell cultures. Only four of twelve cell cultures tested were susceptible to infection and showed clear CPE following inoculation with the cytopathic viruses. In the other cultures, plaques either did not develop or were hazy and indistinct.

Plaque characteristics of viruses. No adaptation to FLB cell cultures was required; all viruses produced plaques in susceptible cells at the first passage. Plaques were observable microscopically, 48 to 72 hours after infection, as small foci of refractile, degenerating cells. The area of affected cells increased with time so that easily countable plaques were apparent 4 to 5 days after infection. Within areas of degenerating cells, apparently healthy cells were often seen (Figure 7.1).

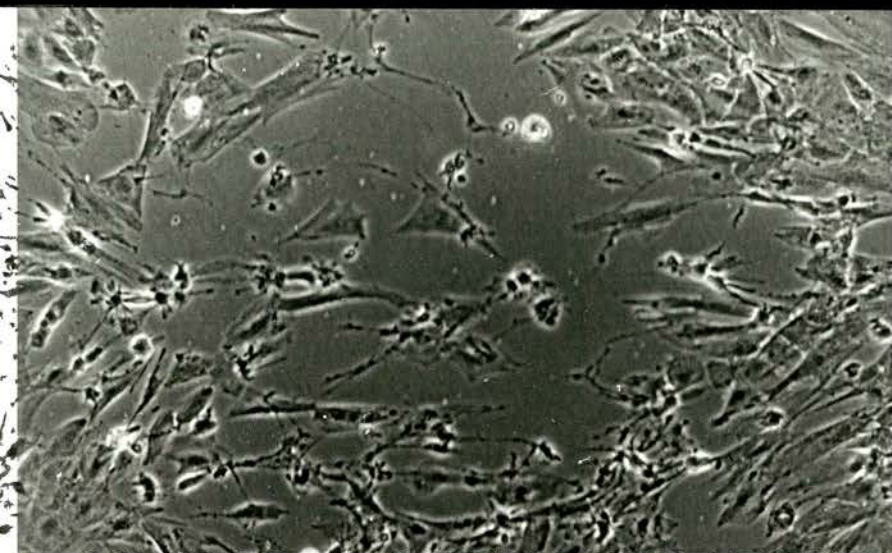
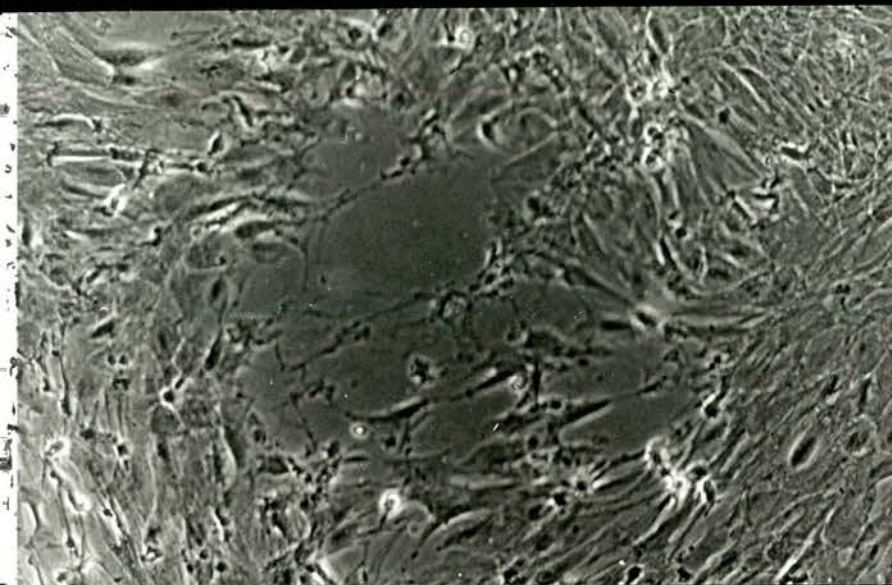
FIGURE 7.1      Appearance of plaques formed by BDV-M in foetal lamb brain (FLB) cells under agarose overlay medium.

Figure 7.1a      Uninfected FLB cells.  
Magnification x 250

Figure 7.1b      Early focus of CPE.      Seventy-two hours after infection.  
Magnification x 120

Figure 7.1c      More extensive CPE.      Ninety-six hours after infection.      Apparently healthy cells can be seen within the area of cell destruction.  
Magnification x 120





Plaque sizes of all the viruses varied from test to test and showed a range of sizes, with the normal distribution of single populations. One virus, BVDV-B816, consistently produced small plaques (Table 7.1; Figure 7.2).

The relationship between the plaque count and the dilution of the inoculum was investigated with 3 of the viruses. Using 6 wells per dilution for each virus, the relationship was shown to be linear over nearly all of the range of dilutions tested, with erratic results occurring only at the end of the range (Table 7.2).

Growth characteristics of viruses. The first evidence of CPE, detected in May-Grunwald Giemsa stained coverslips, was seen 36 hours after infection in cells exposed to BVDV-NADL and BVDV-7446 viruses. Twelve hours later, BVDV-8085 was first seen to cause CPE, but the other two viruses did not cause cell changes until 72 hours after infection. The sequence of CPE development was the same for all viruses.

The earliest changes seen, in a low number of cells, were increased granularity and multiple vacuolation of the cytoplasm. Over the next 4 days an increasing number of cells became affected; many died and became detached from the glass without showing these cytoplasmic changes, but at the end of this time many of the remaining cells showed the multiple cytoplasmic vacuolation very clearly (Figure 7.3). The nuclear changes seen included pyknosis, karyorrhexis and vacuolation; chromatin aggregation occurred in degenerating cells, and when this was surrounded by a clear

TABLE 7.1

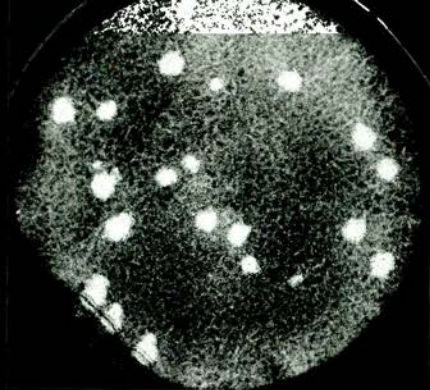
Virus titres, and mean diameters of 50 plaques, of  
5 cytopathic pestiviruses before and after plaque  
purification in foetal lamb brain fibroblasts (FLBF)

Virus	First assay in FLBF		Assay after plaque purification in FLBF	
	Virus Titre (pfu/0.2ml)	Plaque size mm±SD	Virus titre (pfu/0.2ml)	Plaque size mm±SD
BVDV-NADL	6.6	4.4±0.9	4.9	1.6±0.8
BVDV-7446	4.8	4.8±0.9	4.8	3.8±0.9
BVDV-8085	4.7	3.2±0.6	4.9	3.0±0.7
BVDV-B816	5.8	2.2±0.5	4.4	0.8±0.2
BDV-M	4.5	3.3±0.6	5.9	2.3±0.7

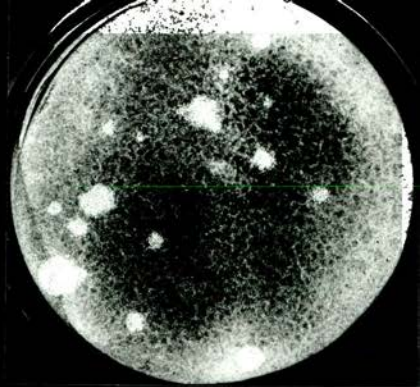
FIGURE 7.2      Plaque characteristics of 5 cytopathic pestiviruses in foetal lamb brain (FLB) cells. Each 55 mm diameter petri dish was inoculated with 0.2 ml of a  $10^{-3}$  dilution of the master stock of plaque purified virus. Actual size.

1. BVDV-NADL
2. BVDV-7446
3. BVDV-8085
4. BVDV-B816
5. BDV-M
6. Uninfected cells

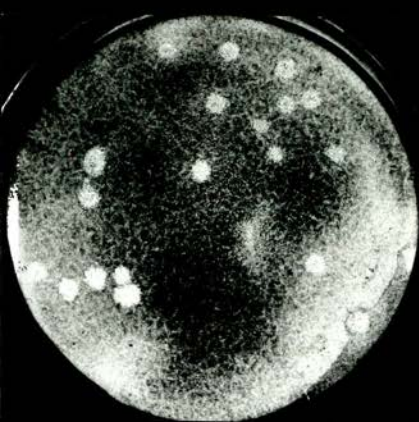




1



2



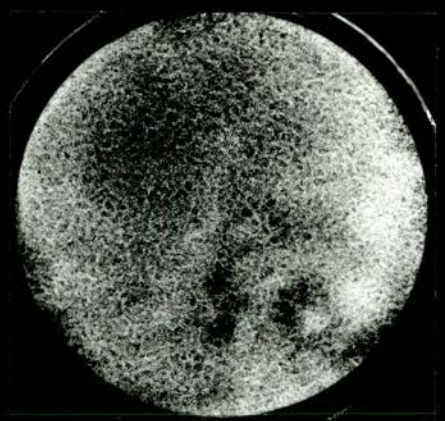
3



4



5



6

TABLE 7.2

THE RELATIONSHIP OF THE NUMBER OF PLAQUE FORMING  
UNITS TO DILUTION OF THREE CYTOPATHIC PESTIVIRUSES

Virus Dilution $\log_{10}$	VIRUS					
	BVDV-NADL		BVDV-8085		BDV-M	
	Mean $\pm$ SE	Titre	Mean $\pm$ SE	Titre	Mean $\pm$ SE	Titre
3.4	20.5 $\pm$ 1.3*	4.7+	22.3 $\pm$ 0.9	4.7	Uncountable	
3.7	12.0 $\pm$ 1.2	4.8	16.5 $\pm$ 0.8	4.9	27.7 $\pm$ 1.0	5.1
4.0	8.6 $\pm$ 0.5	4.9	6.3 $\pm$ 0.2	4.8	15.3 $\pm$ 0.5	5.2
4.3	3.2 $\pm$ 0.5	4.8	4.0 $\pm$ 0.3	4.9	7.6 $\pm$ 0.4	5.2
4.6	1.5 $\pm$ 0.2	4.8	2.7 $\pm$ 0.3	5.0	3.5 $\pm$ 0.4	5.1
4.9	1.0 $\pm$ 0.2	4.9	1.2 $\pm$ 0.2	5.0	1.3 $\pm$ 0.1	5.0
5.2	1.0 $\pm$ 0.2	5.2	0.7 $\pm$ 0.2	5.0	0.3 $\pm$ 0.1	4.7
5.5	0.2 $\pm$ 0.1	4.8	0.0	-	0.2 $\pm$ 0.1	4.8

\* Mean of 6 wells  $\pm$  SE

+ Calculated virus titre expressed as  $\log_{10}$  pfu/0.2 ml

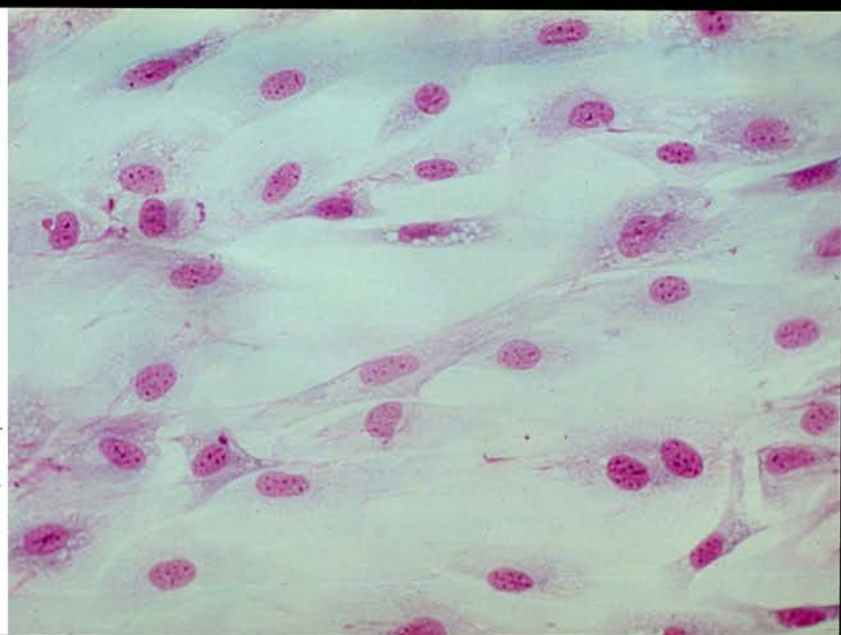
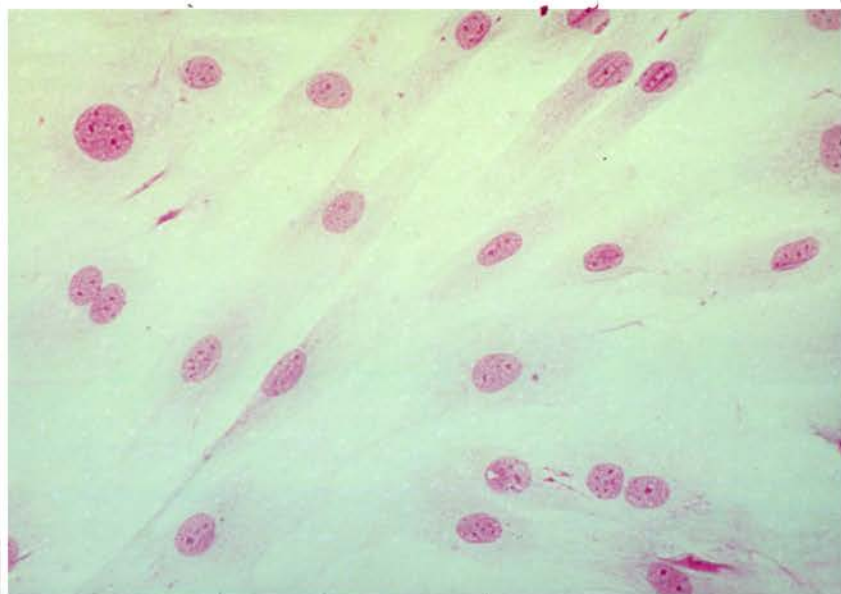
FIGURE 7.3      The development of CPE caused by BDV-M seen in foetal lamb brain (FLB) cells stained with May-Grunwald Giemsa.

Figure 7.3a      Uninfected FLB cells.  
Magnification x 300

Figure 7.3b      Infected FLB cells showing increased granularity of the cytoplasm and intracytoplasmic vacuolation. Sixty hours after infection.  
Magnification x 300

Figure 7.3c      Infected FLB cells showing cell depletion. Remaining cells have multiple cytoplasmic vacuolation and degenerative nuclear changes.  
Magnification x 300





area, the impression was gained of an intranuclear inclusion body. Such structures were only seen late in infection in dying cells.

Specific immunofluorescence was detected only in the cytoplasm of infected cells. The time course for each virus followed that of the stained coverslips, but fluorescence was always seen 24 hours before any CPE was observed. The earliest appearance of fluorescence was in the perinuclear region, usually around the whole perimeter of the nucleus. Diffuse fluorescence spread slowly throughout the cytoplasm, and the whole cytoplasm of degenerating cells usually fluoresced brightly (Figure 7.4).

Growth curves of the 3 viruses tested are shown in Figure 7.5. Despite the different timing of the onset of CPE by the viruses, the growth curves were roughly comparable, with the concentrations of intracellular and extracellular virus being the same during the exponential growth phase. Peak levels of intracellular virus were detected just before the onset of CPE, and were higher than the concentration of extracellular virus at this time. Thereafter the concentration of extracellular virus remained constant over several days, and was always higher than that of the intracellular virus.

Immunofluorescence and neutralisation tests. Both FLK and BEK cells infected with the 5 viruses showed bright intracytoplasmic fluorescence when stained 72 hours after infection. The CPE was advanced in FLK cells at this time, and differed from that in FLB cells. There was more cell clumping and webs of infected cells were seen (Figure 7.6)

FIGURE 7.4      Development of intracellular BDV-M antigen in foetal lamb brain (FLB) cells detected by indirect immunofluorescence.

Figure 7.4a      Fluorescence first detected in the perinuclear region of the cytoplasm of individual cells. Eighteen hours after infection.  
Magnification x 300

Figure 7.4b      Fluorescence has spread to involve more of the cytoplasm and more cells show evidence of viral antigen. Forty-eight hours after infection.  
Magnification x 300

Figure 7.4c      Seventy-two hours after infection, cells show fluorescence throughout the cytoplasm. This is particularly marked in degenerating cells (Top right).  
Magnification x 300

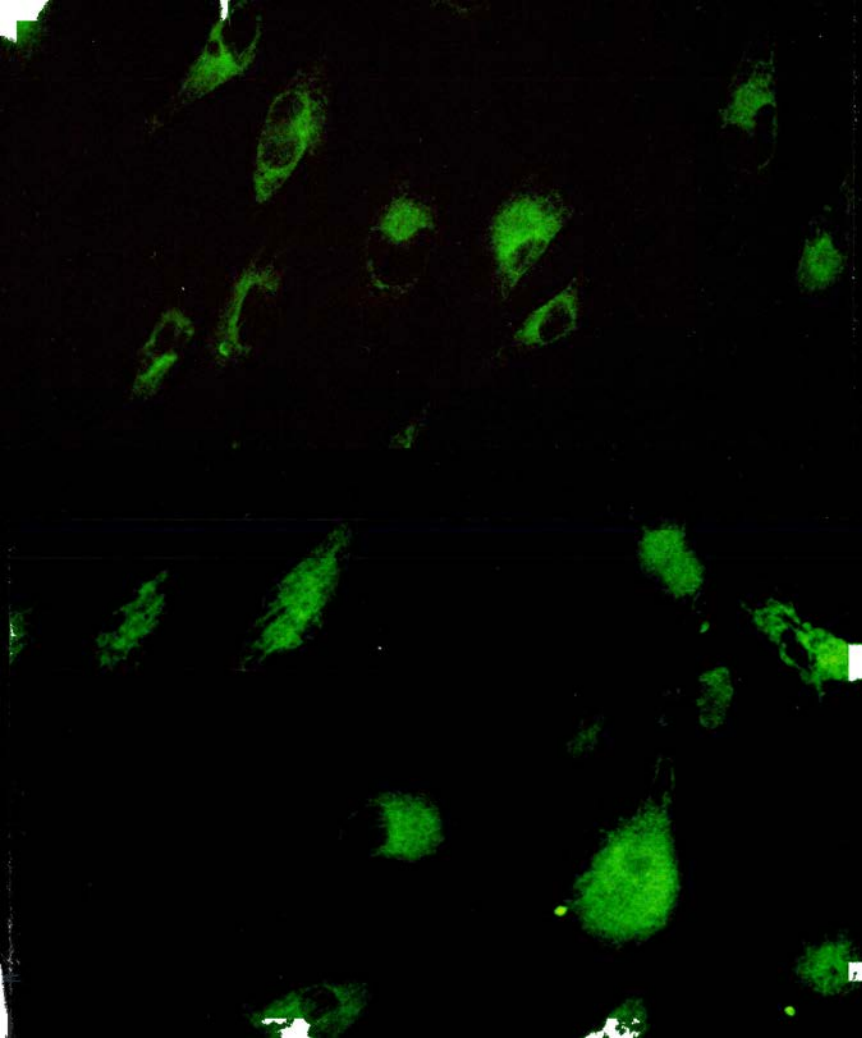


FIGURE 7.5      Growth curves of 3 cytopathic pestiviruses in foetal lamb brain (FLB) cells, infected at a multiplicity of infection of 1.

The concentrations of intracellular ( $\nabla$ --- $\nabla$ ) and extracellular ( $\blacktriangle$ — $\blacktriangle$ ) virus were assayed, and the time at which virus-induced CPE first became obvious was as shown.

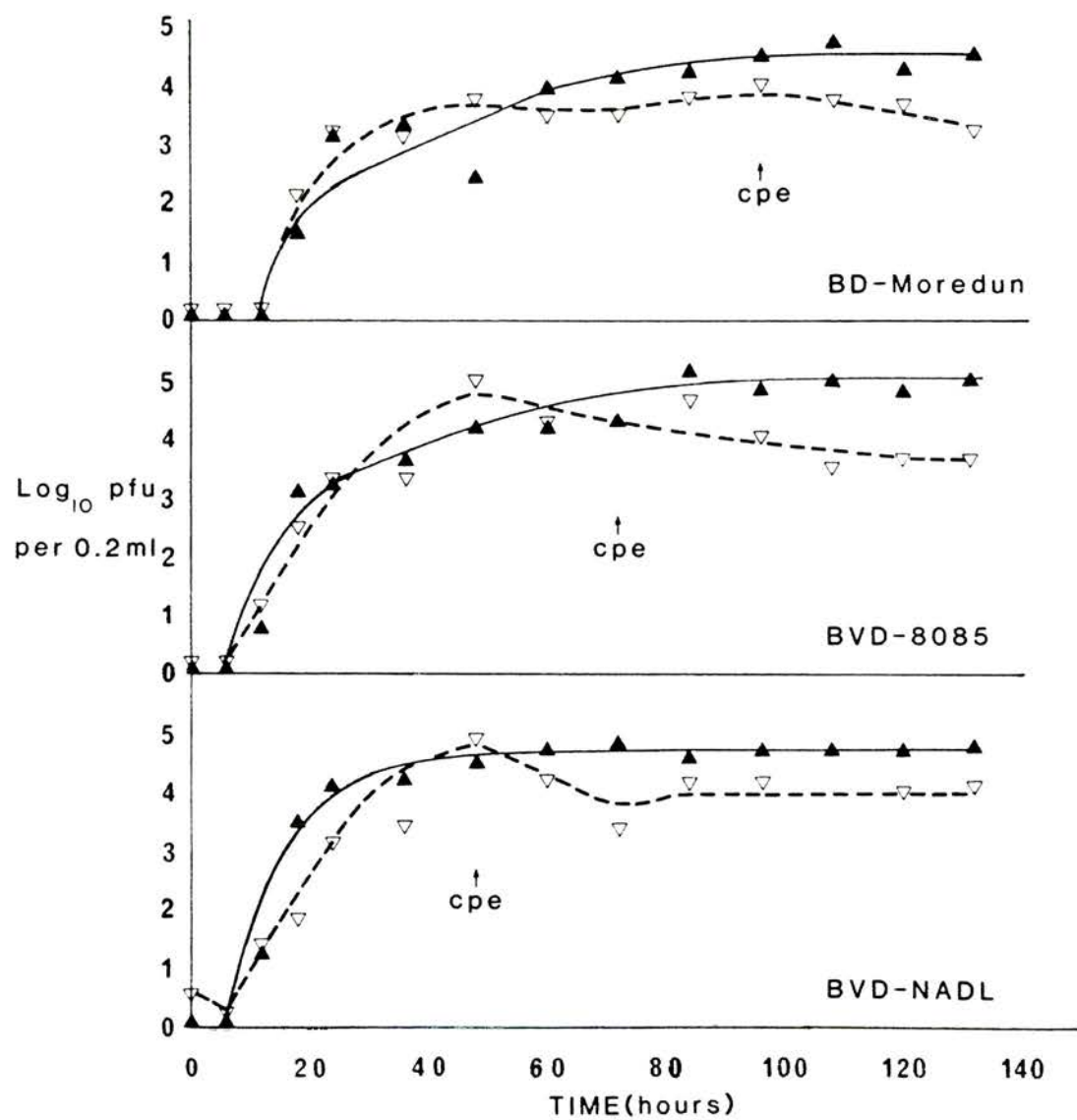
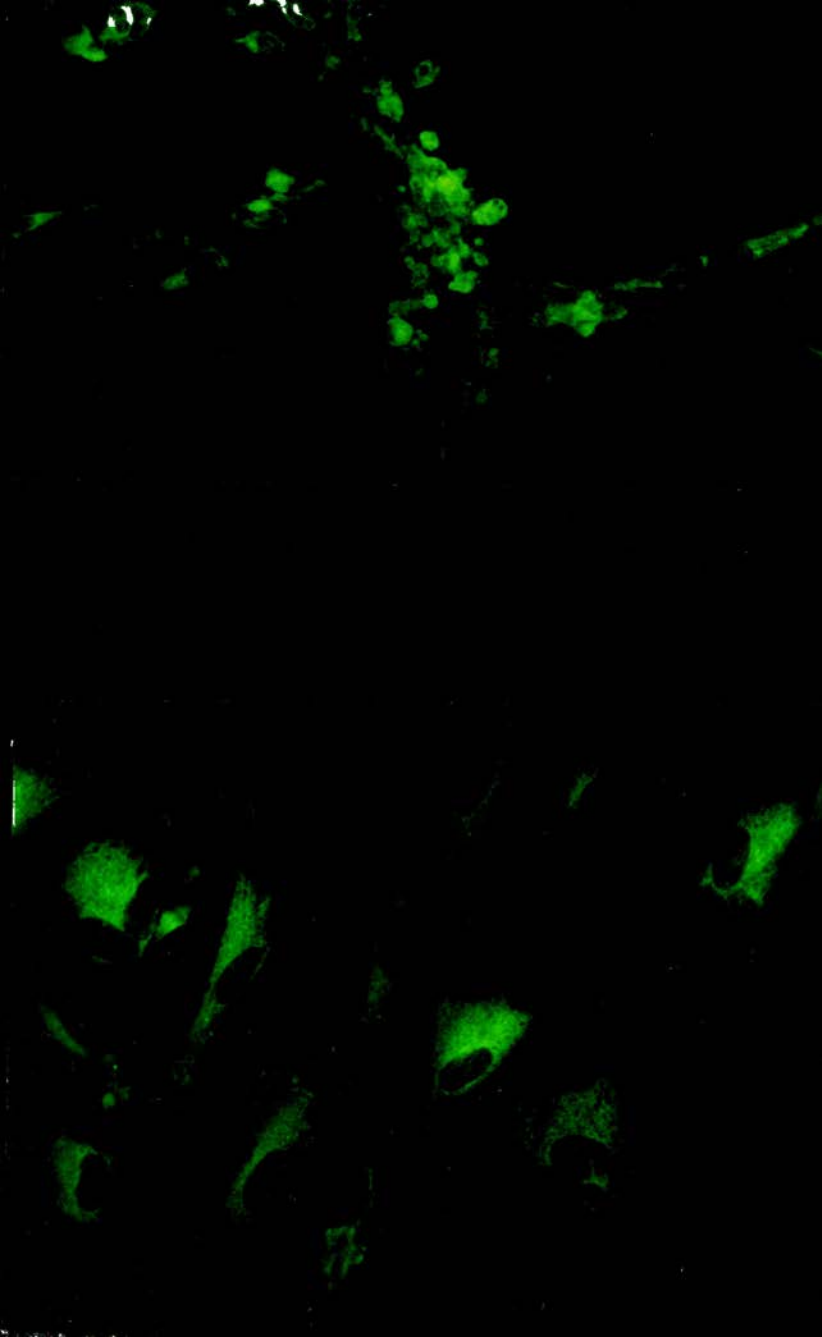




FIGURE 7.6 Indirect immunofluorescence test on foetal lamb kidney (FLK) cells infected with cytopathic BDV-M (Top), and non-cytopathic BD virus(Bottom). Cells stained 72 hours after infection.  
Magnification x 300





The CPE, caused by the 5 viruses was readily neutralised by antisera prepared against the unpurified reference strains (Table 7.3).

Electron microscopy. Negative staining of virus infected cells, in the presence or absence of dilutions of immune sera, did not reveal the presence of any clearly identifiable microorganisms.

Thin sections of FLB cells infected with the 5 viruses produced only meagre evidence of the presence of pestiviruses. In comparison with changes detectable in BE testis cells infected with the unpurified reference strains (Figures 1.2, 1.3, 1.4) , there was less precocious development of the rough endoplasmic reticulum, and only occasional putative virus particles were seen.

Ether and chloroform sensitivity, and filtration studies. The infectivity of all 5 viruses was destroyed by the action of both lipid solvents; only BVDV-B816 showed slight residual infectivity after exposure to ether. In contrast, the infectivity of the bovine enterovirus was unaffected by either chemical. All 5 viruses gave comparable results in the filtration experiment. Only the membrane of 25 nm a.p.d. retained more than 95% of infectious particles (Table 7.4).

Pathogenicity for sheep.

Gnotobiotic lambs. None of the viruses showed any evidence of pathogenicity for gnotobiotic lambs. No clinical disease occurred. The body temperatures of all lambs increased gradually during the first two weeks of life and seven of the lambs reached a temperature plateau around

TABLE 7.3

Neutralisation of plaque purified viruses by  
antisera against unpurified BVDV-NADL and BDV-M

Serum neutralisation index following exposure to		
Virus	BVDV-NADL ANTISERUM (From calf 385)	BDV-M ANTISERUM (Lamb D1479/14)
BVDV-NADL	4.0	1.5
BVDV-7446	2.5	0.5
BVDV-8085	4.0	0.5
BVDV-B816	4.0	0.0
BDV-M	1.0	>3.5

TABLE 7.4

Infectivity titres (pfu/0.2 ml) of plaque purified pestiviruses following exposure to lipid solvents and after filtration through membranes of different average pore diameters (a.p.d.)

Virus	Original titre	After ether	After chlor-form	After filtration through membranes of		
				100nm	50nm	25nm
BVD-NADL	5.1	0.0	<1.0	4.8(50)+4.7(40)	3.6(3)	
BVD-7446	5.1	0.0	<1.0	4.7(40)	4.1(10)	2.9(0.6)
BVD-8085	4.3	0.0	<1.0	4.0(50)	3.4(13)	1.7(0.2)
BVD-B816	4.2	1.5	<1.0	4.1(79)	3.9(50)	2.2(1)
BD-M	4.4	0.0	<1.0	4.3(79)	3.7(20)	1.9(0.3)
G1984 (Enterovirus)	4.5*	4.5	4.5	ND	ND	ND

+ The percentage of virus remaining is shown in brackets.

\*  $\text{Log}_{10}\text{TCID}_{50}$  per 0.2 ml. ND - Not done

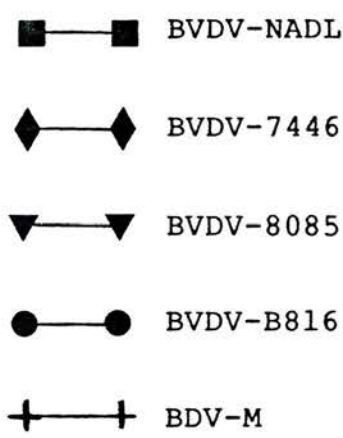
40°C after this rise. Only one day of true pyrexia was recorded: in the first experiment, the lamb infected with BVDV-NADL had a temperature of 40.5°C 7 days after intranasal infection, its temperature on days 6 and 8 being 38.7°C and 39.7°C respectively.

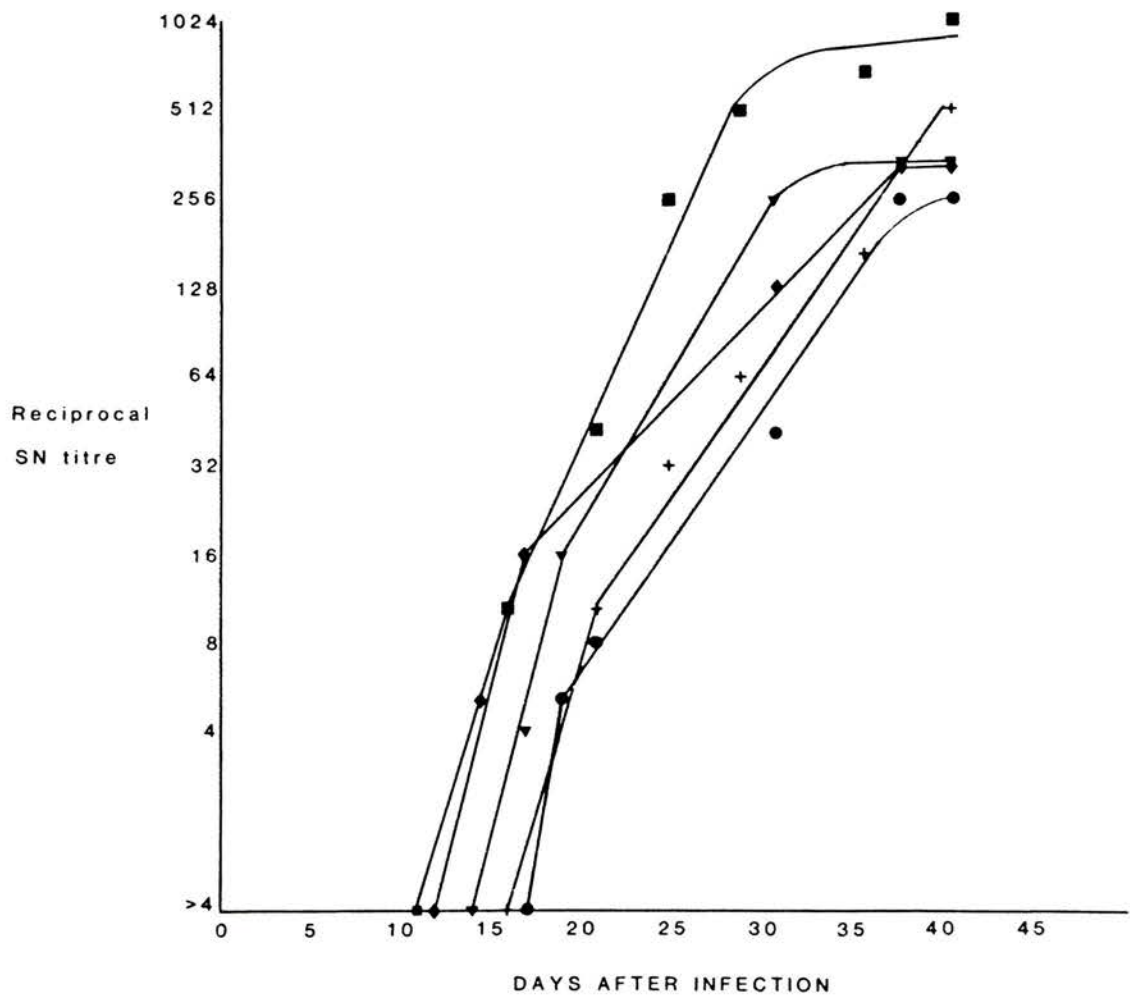
A total of 112 buffy coat samples was tested for the presence of virus, but all samples proved negative. All lambs seroconverted to the virus with which they were infected. In the first experiment only low levels of SN antibody were detectable when the lambs were killed 21 days after infection. All Lambs killed at six weeks, however, had high SN antibody levels at that time, and the rate of antibody development was similar in all the lambs (Figure 7.7).

Pregnant sheep. None of the sheep showed any evidence of malaise following infection with the plaque purified viruses. Examination of the uteri and foetuses 30 days after infection revealed no gross evidence of disease attributable to the pestiviruses. One of the control ewes and one of the ewes infected with BVDV-NADL had recently aborted, but there was no histopathological evidence from the placentae that these abortions were pestivirus-induced.

Histopathological evidence of established pestivirus infection was seen in the placentae of only 6 ewes: two that received BVDV-NADL, two receiving BVDV-8085 and one each of the ewes injected with BDV-M and BVDV-B816. Lesions were small and focal, and were detected only in a low number of the cotyledons examined from each ewe.

FIGURE 7.7      The development of serum neutralising antibodies by gnotobiotic lambs each infected with a different cytopathic pestivirus.







Only two virus isolations were made. Cotyledons were tested from all ewes except the control ewe that aborted, and a cytopathic virus was isolated from one of the ewes injected with BVDV-8085. No viruses were recovered from the foetal blood clots or cerebellum tissue from 23 foetuses, but of 18 foetal spleen/gonad pools tested, one yielded a non-cytopathic pestivirus; this virus-positive pool was from one of two foetuses from a ewe injected with BVDV-NADL.

Low pathogenicity of the plaque purified viruses was also suggested by the low levels of SN antibody present in the sera of the ewes 30 days after infection (Table 7.5). No SN antibodies were detected in the sera of the control ewes or in any of the foetal sera.

#### DISCUSSION

The use of semi-continuous lines of FLB cells to study 5 cytopathic pestiviruses was hampered by the insensitivity of 8 of 12 lines tested. Laude and Gelfi (1979), working with foetal lamb muscle cells, and Rossi et al.(1980) using foetal bovine lung cultures, have also recorded the variable susceptibility of cell lines to pestiviruses. Insensitivity has been attributed to interference by non-cytopathic strains of pestivirus, but failure to demonstrate such contamination in any of the 12 lines studied, suggests that other mechanisms of resistance occur.

In susceptible cells all the viruses formed discrete plaques under agarose-containing medium, enabling purified

TABLE 7.5

Serum neutralising antibody titres of ewes to plaque purified cytopathic pestiviruses, 30 days after infection with those viruses. The SN antibody titres to low passage BVDV-NADL and BDV-M are shown also.

Ewe Number	Injected with	SN ANTIBODY TITRES TO		
		Homologous plaque purified virus	Low passage BVDV- NADL	BDV-M
X431	BVDV-NADL	64	22	<4
Z839		16	11	<4
Z828		<4	<4	<4
Z832	BVDV-7446	11	<4	<4
V889		22	8	<4
X432		45	<4	<4
Z820	BVDV-8085	22	<4	<4
Z826		22	<4	<4
Z830		45	11	<4
V860	BVDV-B816	16	<4	<4
V896		4	<4	<4
X471		<4	<4	<4
X469	BDV-M	22	<4	8
X505		128	4	16
Z824		22	<4	6
X587	Tissue culture fluid	<4*	<4	<4
V862		<4	<4	<4
Z812		<4	<4	<4
Z834		<4	<4	<4

\* Tested against all 5 plaque purified viruses.



stocks to be prepared. Partial characterisation of these stocks showed them to have comparable growth properties in FLB cells, although the rates of CPE development of one of the BVD viruses (BVDV-B816) and of BDV-M were slower than those of the other 3 viruses. Antigen detection by IIFT showed that evidence of virus replication was restricted to the cytoplasm. The perinuclear appearance of fluorescence soon after infection suggests that nuclear contents or the nuclear membrane fulfil a role in viral replication. The failure to detect intranuclear fluorescence, however, makes it unlikely that virus proteins are synthesised at this site. The intranuclear inclusion-bodies were considered to result from chromatin aggregating as the cells were dying. Such inclusion have been described previously in both BVDV and BDV-infected cells (Singh, 1969b; Vantsis et al., 1976). It has recently been suggested that they may represent evidence of the presence of other agents replicating in cells infected with non-cytopathic pestiviruses (Terpstra, 1985; Littlejohns and Walker, 1985). Whilst this possibility cannot be excluded completely, it would seem unlikely since they were seen in cells infected with 5 viruses from very disparate sources.

The limited range of physico-chemical properties tested revealed no significant differences between the 5 viruses, and the results were consistent with earlier findings (Saurat et al., 1972; Harkness and Vantsis, 1982). Immunofluorescence and neutralisation studies showed all the viruses to be antigenically related to the unpurified reference strains.

The infection of gnotobiotic lambs and pregnant ewes revealed that the plaque purified viruses were of low pathogenicity for these animals. Difficulty in recovering virus confirmed that only low levels of viraemia occurred. Nevertheless, there was sufficient viral replication to stimulate the formation of SN antibodies in nearly all the animals. Lack of pathogenicity could have resulted from attenuation following passage in FLB cells, or could have been due to the use of plaque purified virus. There are very few reports of the use of plaque purified pestiviruses for pathogenicity studies. The available evidence, however, suggests that such agents have reduced pathogenicity. In cattle, Nuttall et al., (1980) failed to detect viraemia in calves infected with plaque purified BVDV-NADL, whereas no such difficulty was encountered with calves receiving unpurified NCP virus; the BVDV-NADL being also much less pathogenic for the cattle than the NCP virus. In sheep, the injection of ewes at 54 days gestation with purified cytopathic BDV-M, resulted in the birth of a much higher percentage of normal lambs, compared to ewes given the same passage level of unpurified virus (Vantsis et al., 1976).

There is thus an increasing weight of evidence that purification of cytopathic pestiviruses away from the bulk of the population selects viruses of reduced pathogenicity for normal animals.

CHAPTER 8ANTIGENIC RELATIONSHIPS. COMPARISON OF BRITISH  
ISOLATES OF BVD VIRUS AND BD VIRUS BY  
CROSS-NEUTRALISATION, CROSS-IMMUNOFLUORESCENCE  
AND WESTERN BLOTTING TESTSINTRODUCTION

Antigenic relationships among BVD virus isolates have been studied extensively by cross-immunity and neutralisation tests, and both these procedures confirmed relatedness between early reference strains of CP and NCP isolates from cases of BVD and MD (Gillespie et al., 1960; Gillespie et al., 1961). Neutralisation studies on Italian, German, French and Belgium CP isolates showed that, while all isolates were antigenically related, antigenic differences were distinguishable (Castrucci et al., 1968; Hafez and Liess, 1972; Corthier and Aynaud, 1973). Similar findings were reported by American workers who proposed a classification of strains into 3 serotypes; two represented by the CP virus strains, C24V and NADL, and the third by NCP viruses (Fernelius et al., 1971). Further work on the classification of BVDV strains has not been reported, possibly because Castrucci et al. (1975) demonstrated that serologically different CP strains of BVD viruses were cross-protective in cattle.

Among BD viruses, the BDV-M and Weybridge BD-2 strains have been compared in cross-neutralisation tests, and shown to be related to each other and to BVDV-NADL and to 2 HCV strains. The 2 BD strains were more closely related to each other than to BVD-NADL, and more closely related to the BVD virus than to the HC viruses (Laude and Gelfi, 1979). Weybridge BD-2 has also been compared in



cross-neutralisation tests with 3 strains of BVDV; the reference strains NADL and C24V and a British isolate (81422). The antigenic differences between BD-2 and the BVD virus strains were reported to be no greater than the differences between various other British BVD isolates (Harkness and Vantsis, 1982).

In view of the results of the immunity test in sheep using BVDV-NADL and BDV-M (Chapter 5 Section 3), it was considered worthwhile to investigate further the antigenic relationship between these viruses in cross-neutralisation tests. For further comparison, three cytopathic British isolates were also included in the study, and the neutralisation of NCP pestivirus isolates by antisera against the 2 cytopathic reference strains was investigated. Cross-immunofluorescence studies were also conducted since it has been shown that sera raised against one BVD virus strain may fail to detect another strain growing in culture (Heuschele, 1976).

In the final section of this Chapter, attempts to identify immunogenic proteins of the 5 CP pestiviruses by a "Western Blotting" method are described.

#### MATERIALS AND METHODS

Cell cultures. Semi-continuous cultures of FLB cells free of contaminating NCP pestiviruses, were used between passage levels 5 and 50. They were grown in supplemented Eagle's MEM (Chapter 2).

Viruses. Four cytopathic BVD viruses, and one cytopathic BD virus were used: BVDV-NADL, BVDV-7446, BVDV-8085, BVDV-B816 and BDV-M. Working stocks of the viruses were grown after plaque purification over 3 passages.

Three NCP isolates of BVDV were selected: BVDV-D1787, from a pool of tissues from an aborted foetus, BVDV-E3679, from the lung of a calf dying of pneumonia, and BVDV-G982, from the blood of a persistently infected bull with a sight defect. Three NCP isolates of BDV were chosen: BDV-B1056, from the brain of an experimentally infected lamb, BDV-G1305, from a pool of tissues from a lamb with clinical BD, and BDV-G2048, from the blood of an apparently normal persistently infected lamb. Working stocks of these viruses were prepared following three terminal dilutions in culture.

The details of the time and place of origin of the three CP and six NCP field isolates are summarised in Table 2.1.

Production of antiserum. Antiserum against each of the 5 cytopathic isolates was prepared in a gnotobiotic lamb, as described in Chapter 6. Basically, lambs received 5 ml of virus intranasally the day after birth, 4 ml of virus intravenously at 7 days old, and 2 ml intramuscularly of a four-fold concentrate of virus emulsified with an equal volume of Marcol 52/Montanide 888 at 3 weeks of age. Lambs were exsanguinated under general anaesthesia when they were 6 weeks old, and sera were collected and stored at  $-20^{\circ}\text{C}$  in 5 ml amounts. . The infectivity titres of the viruses were 5.0, 5.0, 5.4, 4.5 and 5.1  $\log_{10}\text{pfu}$  per 0.2 ml for BVDV-NADL, BVDV-7446, BVDV-8085, BVDV-B816 and BDV-M respectively.

A pool of sera from adult Scottish cattle was used as a positive control serum in all neutralisation tests.

Antiserum prepared against low passage BVDV-NADL using autologous cell cultures from calf 368 (Chapter 5) was also used in the Western blotting experiments.

### Serological tests

Microneutralisation tests. The degree of cross-neutralisation between the 5 cytopathic viruses was measured using two-way constant-virus, varying-serum microneutralisation tests. All the serum-virus combinations being compared were tested at one time in the same batch of cells. The results are expressed as the geometric mean values from four tests.

The degree of antigenic relatedness between the viruses was calculated by a slight modification of the method of Archetti and Horsfall (1950) using the formula :-

$$R = \sqrt{r_1 \cdot r_2} \times 100$$

Where  $r_1 = \frac{\text{Heterologous titre (Strain 2)}}{\text{Homologous titre (Strain 1)}}$

and  $r_2 = \frac{\text{Heterologous titre (Strain 1)}}{\text{Homologous titre (Strain 2)}}$

The closer the value of R is to 100 the more closely related are the strains. A value of  $R < 5$  signifies a more than twenty-fold reciprocal difference between the homologous and heterologous titres. Such a difference has been used to define serotypes of rhinoviruses (Kapikian et al., 1967).

The degree of neutralisation of the NCP virus isolates by gnotobiotic lamb antisera raised against BVDV-NADL (Serum ref. No. 705) and BDV-M (No. 704) was measured in constant-serum, varying-virus neutralisation tests. The antisera were diluted so as to contain 40 and 4 SN units, one SN unit being the highest reciprocal dilution of serum which neutralised 100 TCID<sub>50</sub> of homologous virus.



The serum neutralisation index results are expressed as the geometric mean of four results for the 40 SN unit tests and three results for the 4 SN unit tests.

Cross-immunofluorescence tests. In each test, fixed infected and uninfected cells grown on coverslips were exposed to dilutions of all five test sera. The results are expressed as the geometric mean of four tests.

The serological tests were performed as described in Chapter 2.

Western blotting. The guidance of Dr. A.J. Herring and the expert assistance of Mr.N.Inglis are gratefully acknowledged.

Confluent monolayers of FLB cells grown in Sani/Glass (S/G) bottles were washed and infected with virus at a m.o.i. of 1 or with 10 ml of uninfected tissue culture fluid. After 2 hours' adsorption at 37°C, inocula were removed and 50 ml of supplemented maintenance medium added. Forty-eight hours later, the maintenance medium was removed, and cells contained therein sedimented by centrifugation at 2000 x g for 20 minutes at 4°C. Supernatant fluid was discarded and the cells resuspended in 2 ml of PBS. Cells attached to the glass were scraped off with a rubber 'policeman' into 10 ml of PBS, and pooled with those derived from the maintenance medium. Cells were sedimented by centrifugation at 2000 x g for 15 minutes, and the supernatant discarded. The cell pellet was raised in 1 ml of reticulocyte standard buffer (RSB)(10mM NaCl, 10 mM Tris-HCl, 1 mM MgCl<sub>2</sub>: pH 7.5) and kept at 4°C for 10 minutes, after which 110µl of RSB containing 5 per cent NP40 (w/w) was added. Cells were disrupted by gentle

homogenisation in a Griffith's tube, after which the fluid was centrifuged at  $2000 \times g$  for 20 minutes at  $4^{\circ}\text{C}$ . The supernatant was collected, and if not prepared for electrophoresis the same day was stored at  $-20^{\circ}\text{C}$  until use.

Sample preparation, electrophoresis and electroblotting on to nitrocellulose paper were carried out as described by Herring and Sharp (1984). Standard proteins used to measure the molecular weights of the viral proteins were B-galactosidase (130K), phosphorylase A (94K), ovotransferrin (77K), albumin (66K), ovalbumin (45K), chymotrypsinogen A (26K), myoglobin (17K) and cytochrome C (12K).

Strips of nitrocellulose membrane were reacted with 50% (v/v) horse serum in washing buffer (WB), for 1 hour at  $37^{\circ}\text{C}$ , to "blank-out" non-specific binding sites. The strips were then incubated with 1 in 30 dilutions of test sera in 10% (v/v) horse serum in WB for a further hour at room temperature, after which they were washed thoroughly.

Strips were then incubated for 1 hour at room temperature in detection serum diluted in 10% horse serum in WB. The detection antibody was an affinity column purified rabbit anti-sheep  $\text{Fab}_2\text{IgG}$  iodinated to a specific activity of  $3 \times 10^5 \text{cpm}/\mu\text{g}$  and used at a dilution of around  $4 \times 10^5 \text{cpm}/\text{ml}$ . After incubation the strips were washed thoroughly, vacuum dried and mounted on filter paper for autoradiography. This was performed using exposure to Kodak X omat 'S' film with a Dupont 'lightening plus' intensifying screen.

The molecular weights of proteins detected by autoradiography were calculated, from their mobility in the gel, by extrapolation from a standard curve obtained by



plotting mobilities of the marker proteins against their known molecular weights on a semi-logarithmic scale (Weber and Osborn, 1969).

## RESULTS.

Antigenic relationships among 5 CP pestivirus isolates in two-way cross-neutralisation tests. Homologous titres ranged from 2042 to 180 and were higher than, or equivalent, to those obtained with any of the heterologous strains (Table 8.1). The BDV-M reference strain was antigenically distinguishable from the 4 BVD viruses. With R values  $<5$  against the other viruses, BDV-M fulfilled the criterion necessary for it to be considered as a separate serotype. Further evidence that the BDV-M virus is serologically distinct from the BVD viruses infecting Scottish cattle was revealed by the lower titre of the serum pool to this virus compared to the BVD viruses.

Two of the Scottish isolates, BVDV-7446 and BVD-8085, appeared to be closely related to each other, less so to BVD-B816, although none of the differences could be considered significant. Likewise, there was no significant difference between the 3 Scottish field isolates and the BVDV-NADL reference strain. There was, however, a distinct one-sided relationship between BVDV-NADL and sera raised against the other BVD virus isolates.

Neutralisation of NCP BVD and BD virus isolates by antisera raised against the two cytopathic reference strains. All the NCP viruses were neutralised to some extent by both antisera (Table 8.2). The bovine viruses were the same in that they were strongly neutralised by the antiserum

TABLE 8.1

COMPARISON OF 5 CYTOPATHIC PESTIVIRUSES IN TWO-WAY  
CROSS-NEUTRALISATION TESTS

A. Reciprocal neutralisation titres

VIRUS	LAMB ANTISERA RAISED AGAINST				BDV-M	Adult Bovine Conval- escent Pool
	BVDV- NADL	BVDV- 7446	BVDV- 8085	BVDV- B816		
BVDV-NADL	2042	19	69	11	0	331
BVDV-7446	1717	393	605	139	9	938
BVDV-8085	786	181	607	34	4	468
BVDV-B816	197	59	64	180	0	859
BDV-M	18	2	6	2	788	49

B. R Values \*

VIRUS	LAMB ANTISERA RAISED AGAINST				BDV-M
	BVDV- NADL	BVDV- 7446	BVDV- 8085	BVDV- B816	
BVDV-NADL	100				
BVDV-7446	15	100			
BVDV-8085	21	68	100		
BVDV-B816	7.5	34	14	100	
BDV-M	0	1	1	0	100

$$* R = \sqrt{r_1 \cdot r_2} \times 100$$

Where  $r_1 = \frac{\text{Heterologous titre (Strain 2)}}{\text{Homologous titre (Strain 1)}}$

and  $r_2 = \frac{\text{Heterologous titre (Strain 1)}}{\text{Homologous titre (Strain 2)}}$

TABLE 8.2

NEUTRALISATION OF NON-CYTOPATHIC BVD AND BD  
VIRUSES BY TWO DILUTIONS OF ANTISERA RAISED  
AGAINST THE CYTOPATHIC REFERENCE STRAINS,  
BVDV-NADL AND BDV-M

VIRUS	40 SN UNITS OF SERUM		4 SN UNITS OF SERUM	
	705 Against BVDV-NADL	704 Against BDV-M	705 Against BVDV-NADL	704 Against BDV-M
BVDV-D1787	3.6+	0.9	1.9	0.2
BVDV-E3679	3.1	0.6	1.5	0.1
BVDV-G982	3.6	1.2	2.0	0.5
BDV-B1056	2.7	1.8	1.6	0.2
BDV-G1305	1.1	2.8	0.6	1.4
BDV-G2048	0.9	2.5	0.4	1.0

+  $\text{Log}_{10}$  serum neutralisation index.

against BVDV-NADL, less so by the BDV-M antiserum; the differences between the neutralisation indices were of the order of 200 to 500 using 40 SN units of antisera, but 10-fold less than this with the more dilute sera.

The results with the NCP ovine viruses were not so clear-cut. Two of the isolates (BDV-G1305 and BDV-G2048) were neutralised more effectively by the BDV-M antiserum, while the third (BDV-B1056) appeared to be more closely related to BVDV-NADL.

Antigenic relationships among 5 CP pestivirus isolates in two-way cross-immunofluorescence tests. No significant differences between the viruses were detected in these tests. There was never more than a 3-fold difference between the highest dilution at which any serum detected fluorescence in cells infected with its homologous virus compared to the heterologous viruses. Two sera, those raised against BVDV-NADL and BVDV-B816, were able to detect fluorescence at higher dilutions than the other 3 sera. (Table 8.3).

Antigenic relationships among 5 CP pestiviruses by Western blotting. When sera were reacted against preparations of uninfected cells several bands were detected. Long exposure of the autoradiograph increased the number and intensity of these bands (Figure 8.1). The majority of these bands were also present when sera were reacted with infected cell extracts, but two additional bands could also be detected. The specificity of these bands was confirmed by reducing exposure times and by the incubation, before use, of test sera with strips of membrane to which uninfected cell proteins were bound (Figures 8.2 to 8.5).

TABLE 8.3

COMPARISON OF 5 CYTOPATHIC PESTIVIRUSES IN  
TWO-WAY CROSS-IMMUNOFLUORESCENCE TESTS

VIRUS	LAMB ANTISERA RAISED AGAINST				
	BVDV- NADL	BVDV- 7446	BVDV- 8085	BVDV- B816	BDV-M
BVDV-NADL	722*	90	139	331	83
BVDV-7446	1214	139	197	278	70
BVDV-8085	607	90	180	468	139
BVDV-B816	724	107	128	512	152
BDV-M	215	59	90	152	215

\* Results are expressed as the reciprocal of the highest dilution of antiserum that produced clear specific immunofluorescence in an IIFT.

FIGURE 8.1      Uninfected      FLB      cell      extracts      on  
nitrocellulose membrane strips were reacted  
with      preinfection      (Odd numbers)      and  
convalescent (Even numbers) test sera :-

1,2	Calf	anti BVDV-NADL
3,4	Lamb	anti BVDV-NADL
5,6	Lamb	anti BDV-M
7,8	Lamb	anti BVDV-7446
9,10	Lamb	anti BVDV-8085
11,12	Lamb	anti BVDV-B816

Sixty-four hour exposure of the  
autoradiograph revealed several bands in the  
even numbered strips, many of which were also  
visible faintly in the strips exposed to  
preinfection sera. This is most noticeable in  
strips 1 and 2.

FIGURE 8.2      Extracts      from      FLB      cells      infected      with  
BVDV-NADL on nitrocellulose membrane strips  
were reacted with preinfection (Odd numbers)  
and convalescent (Even numbers) test sera:-

1,2	Calf	anti BVDV-NADL
3,4	Lamb	anti BDV-M
5,6	Lamb	anti BVDV-NADL
7,8	Lamb	anti BVDV-7446
9,10	Lamb	anti BVDV-8085
11,12	Lamb	anti BVDV-B816

Twenty-four hour exposure. The 78K virus  
band is detected by all convalescent sera,  
but the 35K protein is only visible in strips  
exposed to anti BVDV sera, being particularly  
strong with the homologous serum in strip 6.

1 2 3

5

6

7

8

9

10

11

12

Mol. Wt.  
Markers

— 130K

— 93K

— 77K

— 66K

— 45K

— 26K

— 17K

1

2

3

4

5

6

7

8

9

10

11

12

← 78 K

← 35 K



FIGURE 8.3      Extracts from FLB cells infected with BVDV-7446 on nitrocellulose membrane strips were reacted with preinfection (Odd numbers) and convalescent (Even numbers) test sera :-

1,2	Calf	anti BVDV-NADL
3,4	Lamb	anti BVDV-NADL
5,6	Lamb	anti BDV-M
7,8	Lamb	anti BVDV-7446
9,10	Lamb	anti BVDV-8085
11,12	Lamb	anti BVDV-B816

The 78K virus band is visible with all convalescent lamb sera, but the 35K virus protein is only detected by one of the anti BVDV sera.

Figure 8.4      Extracts from FLB cells infected with BVDV-8085 on nitrocellulose membrane strips were reacted with preinfection (Odd numbers) and convalescent (Even numbers) test sera:-

1,2	Calf	anti BVDV-NADL
3,4	Lamb	anti BVDV-NADL
5,6	Lamb	anti BDV-M
7,8	Lamb	anti BVDV-7446
9,10	Lamb	anti BVDV-8085
11,12	Lamb	anti BVDV-B816

The 78K virus band is visible with all the convalescent sera, but the 35K protein is only visible in strips 4 and 10

1 2 3 4 5 6 7 8 9 10 11 12

← 78K

← 35K

1 2 3 4 5 6 7 8 9 10 11 12

← 78K

← 35K

FIGURE 8.5      Extracts from FLB cells infected with BVDV-B816 on nitrocellulose membrane strips were reacted with preinfection (Odd numbers) and convalescent (Even numbers) test sera :-

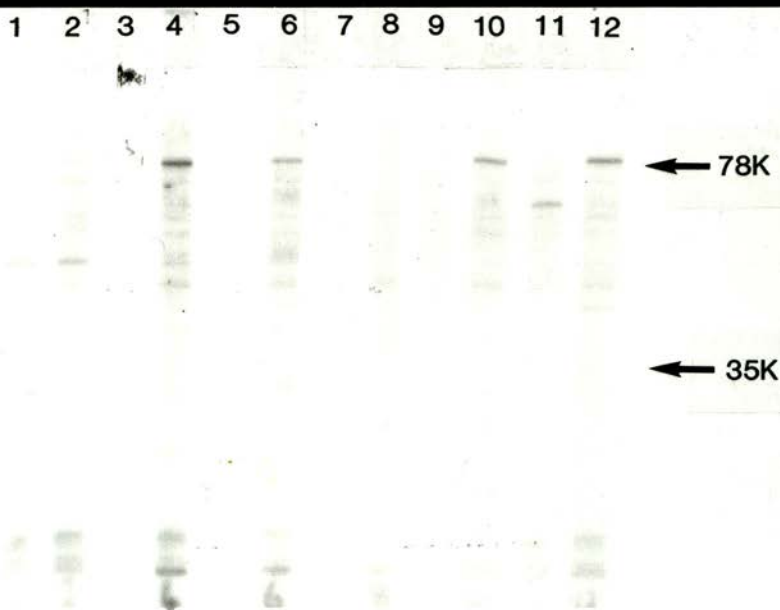
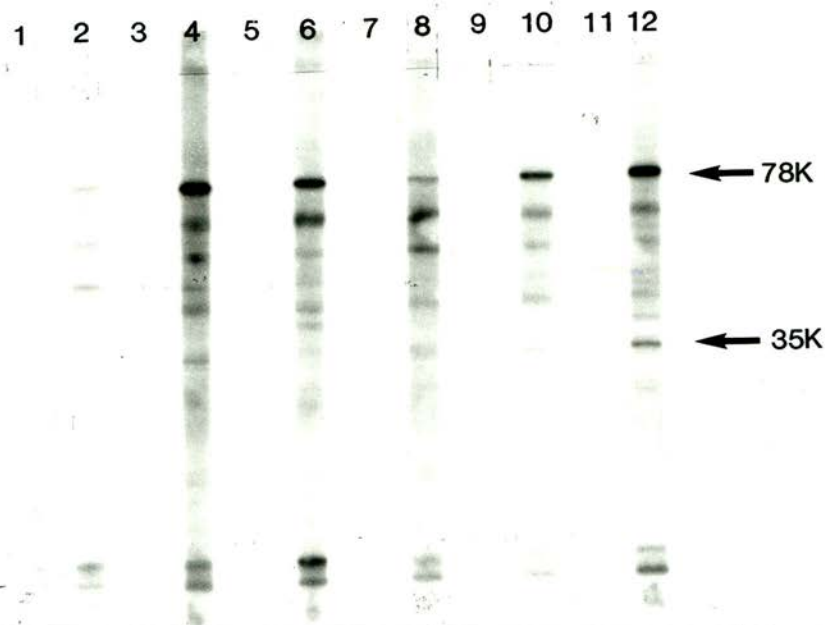
1,2	Calf	anti BVDV-NADL
3,4	Lamb	anti BVDV-NADL
5,6	Lamb	anti BDV-M
7,8	Lamb	anti BVDV-7446
9,10	Lamb	anti BVDV-8085
11,12	Lamb	anti BVDV-B816

The 78K virus band is clear with all convalescent sera. The 35K virus protein is visible most clearly in strip 12, the homologous antiserum.

Figure 8.6      Extracts from FLB cells infected with BDV-M on nitrocellulose membrane strips were reacted with preinfection (Odd numbers) and convalescent (Even numbers) test sera:-

1,2	Calf	anti BVDV-NADL
3,4	Lamb	anti BVDV-NADL
5,6	Lamb	anti BDV-M
7,8	Lamb	anti BVDV-7446
9,10	Lamb	anti BVDV-8085
11,12	Lamb	anti BVDV-B816

The 78K virus band is visible with all but one of the convalescent sera, but there is no evidence of a 35K virus protein.



Two viral proteins were detected in preparations of the 4 plaque purified BVD viruses by gnotobiotic lamb antisera against these viruses and by calf serum against low passage BVDV-NADL. The same two proteins were detected when low passage BVDV-NADL, which had not been plaque purified, was examined with the same sera. The mean molecular weights of the two proteins were 78,000 and 35,000 (Table 8.4).

Antiserum against BDV-M, however, always detected only the higher molecular weight protein in BVD Virus preparations. When BDV-M was used as antigen, the high molecular weight protein was detected by all the sera, but no low molecular weight protein was detected even by the BDV-M antiserum (Figure 8.6)

#### DISCUSSION

Results of the cross-neutralisation tests between the CP viruses revealed that BDV-M was antigenically distinguishable from the four BVD viruses. This extends the finding of Vantsis et al. (1976), that BDV-M and BVDV-NADL were serologically distinct. In that report, sera from sheep injected with BDV-M which had been purified by differential centrifugation and terminal dilution showed the least cross-reactivity with BVDV-NADL. Similarly the results reported here demonstrated that sera raised against plaque purified viruses were even more discriminatory than those raised against unpurified viruses.

Little is known of the epidemiology of BVD and BD viruses. The preliminary evidence obtained from these studies showed that, despite originating from different disease syndromes from animals in different locations, the

TABLE 8.4

MOLECULAR WEIGHTS OF PROTEINS OF 5 CYTOPATHIC  
PESTIVIRUSES DETECTED BY WESTERN BLOTTING

VIRUS	HIGH MOLECULAR WEIGHT PROTEIN	LOW MOLECULAR WEIGHT PROTEIN
BVDV-NADL *	77,600	37,200
BVDV-NADL	78,500	34,300
BVDV-7446	78,500	35,100
BVDV-8085	76,700	34,300
BVDV-B816	78,500	35,100
BDV-M	81,300	ND
Means	78,000	35,000

\* Low passage virus, not plaque purified. All the other viruses were plaque purified stocks.

ND, Not detected

6 BVD viruses appeared to be closely related antigenically. Two of the NCP ovine viruses were neutralised better by the antiserum against BDV-M than that raised against BVDV-NADL, suggesting that sheep pestiviruses may have evolved separately from those in cattle. The third NCP ovine virus, however, was more closely related to BVDV-NADL. This isolate (BDV-B1056) was chosen deliberately as it had originated from an outbreak of border disease in which the ewes had had higher SN titres to BVDV-NADL than to BDV-M (Vantsis et al., 1980). This virus may have originated from cattle or the range of antigenicity among BD viruses may be wider than that among BVD viruses.

The cross-immunofluorescence tests showed that all the viruses shared a common antigen that was detected by this method, and that this antigen was different from the one determining the degree of cross-neutralisation between the isolates. A possible explanation of this finding was provided by the Western blotting studies which were undertaken to examine the protein composition of the 5 cytopathic pestiviruses.

The difficulty of obtaining good yields of virus free of contaminating host cell proteins is reflected in the current confusion over the protein composition of pestiviruses. There are five reports, in all of which cytopathic viruses, usually BVDV-NADL, were studied. The first report described 4 virus-specific proteins, two of which migrated heterogeneously, having molecular weights of 110,000 - 93,000: 110K - 93K, 70K, 59K - 50K and 25K (Pritchett and Zee, 1975). A few years later, Matthaeus (1979) described 3 viral polypeptides with molecular



weights of 57K, 44K and 34K, the two larger proteins being glycosylated. Coria et al. (1983) using the Singer strain, detected 4 proteins with molecular weights of 75K, 66K, 54K and 26K, of which the 75K and 54K proteins were glycosylated. Close agreement existed between the other two groups of workers who both described 5 proteins of molecular weights 115K, 80K, 54K (55K), 45K and 35K (38K) (Akkina, 1983; Purchio et al., 1984). The 115K and 80K proteins were shown to be related and the 54K (55K) and 45K proteins were shown to be glycosylated.

Considering the similar findings in the last 2 reports and their agreement with the work of Matthaeus (1979), it would seem likely that there are 3 viral structural proteins of molecular weights 57K to 54K (VP1), 45K or 44K (VP2) and 38K to 34K (VP3)., with the larger two being glycosylated. There is also good evidence for a larger viral protein 70K to 80K, but whether this is structural is not yet clear.

The Western blotting procedure described in this Chapter clearly failed to detect the 2 glycosylated structural proteins, VP1 and VP2, Although they may have been obscured by the presence of host cell protein, it is more likely that they were not recognised by the 'early' immune serum employed, possibly because of viral protein denaturation during sample preparation. The 77K to 81K viral protein detected in all our virus preparations almost certainly corresponds to the larger BVD viral protein described by other workers, and probably represents the soluble antigen common to all members of the pestivirus group (Matthaeus, 1980). This could also help to explain

why antiserum against BVDV-7446 produced only a weak reaction with this protein. It has been shown that the quantity of soluble antigen produced may vary considerably between BVD virus isolates (McClurkin and Coria, 1978).

In the experiments reported in this Chapter, the small 35K protein, which was detected only in BVD virus preparations by antisera against BVD viruses, represented VP3. This protein has previously been suspected as being type-specific, since VP3 from BVD virus was not precipitated by HCV antibodies (Matthaeus, 1981). A similar situation would, therefore, appear to exist between the BVD and BD viruses employed in our experiments, although this will not be proved until the 35K protein has been detected in BDV-M preparations by homologous antisera. Further credibility has been given to the importance of the low molecular weight protein by a recent report that a 37K polypeptide is the major surface protein of BVD virus (Zee and Chu, 1984).

In conclusion, the tests described in this Chapter did not detect major antigenic differences between the 7 BVD viruses examined. The cytopathic reference strain of BD virus, however, was antigenically distinct from the bovine viruses in the cross-neutralisation tests, and this distinction could be the result of structural differences in the 35K viral protein.

CHAPTER 9GENERAL DISCUSSION AND CONCLUSIONS

Aspects of the pathogenic and antigenic relationships between BVD and BD viruses have been studied.

At the outset of this work it was well established that both viruses shared the ability to cause congenital infections following the infection of susceptible pregnant cattle and sheep. In a comprehensive review of congenital infections caused by pestiviruses and other non-arthropod borne togaviruses, Van Oirschot (1983) emphasised the ability of the pestiviruses to establish lifelong persistent infections in cattle, sheep and pigs which had survived an early intrauterine infection. These animals shed virus continuously and were likened by Van Oirschot to "the 'Trojan Horse' that surreptitiously introduces an army of pestiviruses into animal populations".

The existence of such animals has serious implications for strategies to control pestivirus-induced diseases.

Among cattle the occurrence of persistently infected animals has often been demonstrated, with or without signs of unthriftiness (Malmquist, 1968; Coria and McClurkin, 1978b; Done et al., 1980; Straver et al., 1983). What the prevalence is of such animals is difficult to ascertain. A survey of apparently healthy slaughterhouse cattle in Denmark detected 12 viraemic animals in 1332 animals tested, but in herds with recent outbreaks of BVDV-related disease 38 of 363 apparently healthy cattle were viraemic

(Meyling, 1984). In this present work, indirect evidence of the prevalence of persistently infected cattle in Scotland was gained from the results of testing secondary BEK cells derived from abattoir fetuses for the presence of BVD virus. The contamination rate of 13.3% indicated a high incidence of intrauterine exposure of calves to the virus, a proportion of which would have been born persistently infected (Chapter 3).

Among sheep, persistently infected lambs frequently have signs of clinical BD but this is not true of animals infected with some virus strains (Westbury et al., 1979; Terpstra, 1981). There is little evidence on which to base an estimate of the number of persistently infected sheep in any population. A serological survey has shown the prevalence of antibodies to pestiviruses among sheep in England and Wales to vary from region to region (Harkness et al., 1978). In Scotland only 4% of sheep have antibodies to BDV-M (Vantsis et al., 1979). In spite of this apparent low prevalence of infection, it is of interest that the results in Chapter 3 show that no fewer than 1.9% of the FLK cells prepared from local abattoir fetuses were contaminated with a non-cytopathic pestivirus. Thus the risk of transmission of infection to susceptible animals is present in many flocks.

Of the 3 types of viral persistence identified by Mims (1982), pestiviruses clearly fit into the type offering "the ultimate insult to the host immune defences by replicating and being continuously shed to the outside world". Pestiviruses can thus provoke and maintain a state

of persistence which has far-reaching consequences for the infected individual, and for the population as a whole.

Although the mechanisms responsible for inducing and maintaining persistent pestivirus infections are not fully understood, they represent a fascinating biological phenomenon. It is reasonable to suggest that some degree of immune tolerance by the host must exist and, in order to test the specificity of this tolerance, the experiments described in Chapter 4 were undertaken. These showed not only that lambs persistently infected with BDV-M were very susceptible to superinfection with a tissue culture stock of the same virus but that seven of eight RHS lambs suffered severe disease which, in some respects, resembled natural cases of bovine MD. Two important findings were that animals dying from the infection failed to mount an effective humoral immune response to the virus, and that cytopathic BDV-M was readily recoverable from their tissues. The mechanisms involved were obscure but a justifiable practical conclusion was that the mucosal disease-like syndrome could be precipitated only in persistently infected lambs since the same virus given to normal lambs produced no clinical disease (Chapter 4; Barlow et al., 1983; Gardiner et al., 1983).

Similar findings have recently been reported in cattle (Roeder and Drew, 1984; Brownlie et al., 1984; Nagele, 1984; Bolin et al., 1985b). Thus the prediction of Malmquist (1968), that many cases of MD could result from immunological tolerance has been substantiated, and the early proposal of Liess et al. (1974), that MD is not an

acute viral infection, has also been largely vindicated. Moreover, the enigma of why only a small number of cattle in a group ever succumb to MD and why the disease can rarely be reproduced is also partially explained.

Now that it is clear that persistent pestiviraemia in cattle and sheep arising from early intrauterine exposure is an essential prerequisite for the development of MD, or the equivalent disease in sheep, further attention ought to be focused on the factors that can trigger clinical illness.

The available evidence is limited, but under experimental conditions at least, the disease can be precipitated by superinfection with some other pestiviruses. This was seen clearly in the experiments reported in Chapters 4 and 6. No other comparable studies with sheep have yet been documented, although Terpstra (1985) failed to induce disease in persistently infected sheep superinfected with homologous or heterologous Dutch isolates of BDV. The results reported in Chapters 4 and 6 also indicate that the outcome of experimental superinfections depends on the nature of the inoculum and the status of the persistently infected sheep. Because high passage virus and experimentally produced RHS were used in the first experiments (Chapter 4) while low passage virus and recovered field cases were used in the second experiment (Chapter 6) no direct comparisons are valid.

The results of the experiments reported in Chapter 4 showed that sheep, persistently infected with virus contained in the IIB brain pool, developed disease when

superinfected with a high tissue culture pass of BDV-M derived from that pool, but not when superinfected with a high tissue culture pass of BVDV-NADL. This result could have been due to differences in the pathogenicity of the two viruses, although it is considered more likely that the antigenic disparity accounted for the differing responses. One conclusion could, therefore, be that, in order to precipitate disease, the superinfecting virus must be antigenically closely related to the virus causing the persistent infection.

Antigenic diversity between the persisting and superinfecting virus may have contributed to the different responses of the two groups of field cases of RHS lambs reported in Chapter 6. What became clear from that experiment, however, was that the outcome of superinfection experiments could differ between groups of animals, and even amongst animals from the same outbreak. Also, it became clear that persistently infected animals injected with live pestivirus could seroconvert to that virus but remain a source of infection.

One further point arising from the sheep experiments that merits discussion relates to the development of a spontaneous mucosal disease-like syndrome in the persistently infected twins, 1261 and 1262 (Chapter 5 section 4). The clinical symptoms, and pathological and virological results were wholly comparable to those seen in the superinfected sheep in Chapter 4, and yet there had been no opportunity for superinfection to occur. The disease appeared in the twins as the levels of



maternally-derived antibody became undetectable, but other persistently infected lambs in the same pen were not affected. The major difference between the lambs showing clinical signs of disease and their pen-mates was that they had been sired by a different tup. Because host genetic factors have an important influence on other aspects of BD virus infections (Barlow et al., 1979) it is not unreasonable to suppose that they also play a part in the fate of persistently infected lambs.

The deliberate reproduction of MD in experimental cattle has been reported recently. During field investigations of cases of MD, Brownlie et al. (1984) isolated NCP BVD virus from the blood of apparently healthy persistently infected cattle, and a mixture of NCP and CP viruses from animals dying of MD. Cytopathic virus was found at high levels in gut tissue and could be separated from the NCP virus by plaque purification. Also when two persistently infected cattle were infected intranasally with the CP virus they both died of acute MD within 2 to 3 weeks, whereas normal cattle receiving the same inoculum, remained healthy. These findings led the authors to propose that "clinical mucosal disease in cattle requires a persistent infection with NCP BVDV and a subsequent infection with CP virus". This hypothesis is helpful, and is consistent with their findings, but is unlikely to embrace every situation in which BVDV infection results in MD.

In another recent report, Bolin et al. (1985b) described the production of severe MD in persistently infected cattle, following the intravenous injection of CP virus.

These findings in cattle imply that the distinction between NCP and CP pestiviruses is critical. Regrettably, the CP characteristic is insufficiently defined and is not necessarily an intrinsic viral property. While some strains maintain their cytopathogenicity in a wide range of host tissues, the cytopathogenicity of others changes during passage in the same or in different host systems (Fernelius et al., 1969a). This feature of pestivirus behaviour was demonstrated clearly by the results in Chapter 3 which showed that BDV-M was highly cytopathic for ovine cells but markedly less so for bovine cells. Nevertheless, high titres of BDV-M were produced by BE testis cells, although it could be detected only by its CPE in sensitive FLK cells. On the other hand, both BDV-M and BVDV-NADL failed to induce CPE in porcine cells, although infectious virus was produced.

A further implication of the recent work in cattle is that every laboratory isolate of cytopathic BVDV has to be a heterogeneous mixture of NCP and CP virus, a supposition recently endorsed by the demonstration of both types of virus in 28 of 38 bovine spleens from diseased animals (McClurkin et al., 1985). Passage of these two populations in tissue culture can only lead to variable proportions of each virus at every pass level since NCP virus interferes with CP virus (Gillespie et al., 1962) and CP virus grows faster than NCP virus (Nuttall, 1980).

The idea of at least two classes of virus within any given pestivirus population would help to explain the capricious growth of some CP isolates and the reports of

NCP viruses causing a CPE under certain conditions (Straver,1971; Laude and Gelfi,1979). In one such account the fact that BVDV and BDV isolates, which were NCP in conventional fluid overlay cultures, produced plaques at high dilutions when titrated under agar overlay, strongly suggested that an interference phenomenon was involved (Evermann et al.,1981).

Failure to titrate CP and NCP viruses within our experimentally infected sheep detracts from the findings reported in this thesis, but nevertheless a summary of the results allows some conclusions to be reached. From the tissues of lambs dying after superinfection with 28th passage BDV-M, virtually all isolates were cytopathic. The infection of ewes at 52 days gestation with low passage BDV-M resulted in the recovery of both CP and NCP viruses from foetuses, but only NCP virus was recovered from the precolostral blood of live lambs. Two of these lambs suffered from an MD-like disease nine weeks later and when they were killed in extremis CP virus was isolated from virtually all their tissues. From normal lambs injected with low pass virus, transient viraemias were detected but only NCP virus was recovered. The same virus given to RHS lambs produced transient viraemias of CP virus against the background of consistently recoverable NCP virus. CP virus was recovered from many tissues of the animals in one of the groups of RHS lambs, but not from any of the other group. When BDV-M and the 4 BVD viruses were plaque purified and used to infect gnotobiotic lambs and pregnant ewes, there was an almost total failure to recover virus from blood or placental and foetal tissues.

If the existence of both CP and NCP virus within any given pestivirus population is accepted, the results summarised above could be explained as follows. The 28th passage virus contained a high proportion of CP virus following prolonged cultivation in vitro making it a potent inducer of the MD-like syndrome in persistently infected lambs. The low passage BD virus contained only a slightly higher proportion of CP to NCP virus. The combination was highly pathogenic for foetuses, which were infected predominantly with NCP virus. In normal lambs replication of the CP virus was restricted so that only NCP virus was detectable in the blood. In the RHS lambs CP virus caused a transient viraemia in both groups, one of which eliminated the virus before it became established at tissue sites. When the CP virus was plaque purified it appeared to be defective in vivo. Limited replication must have occurred since SN antibody was produced and minor pathological lesions were present in the placenta of some ewes. Virus levels, however, remained below detectable limits, apart from two isolations.

The idea that the CP component of a pestivirus population could be defective in vivo is not new. The possibility was discussed when normal lambs were born to ewes infected with purified BDV-M during early gestation (Vantsis et al, 1976). These authors likened their findings to the defectiveness of Rous sarcoma virus, and the analogy is worth pursuing. In that system, Rous associated virus (RAV) is an avian leukosis virus isolated from stocks of Rous sarcoma virus (RSV). The two viruses are

indistinguishable in size and structure; they have the same sensitivity to thermal inactivation, and have the same buoyant density in rubidium chloride. They are indistinguishable in neutralisation tests and have parallel growth curves (Hanafusa et al., 1964). The viruses do, however, differ markedly in biological effect, since only RSV but not RAV produces foci of transformed cells in tissue culture and sarcomas in chickens. It has been concluded that RSV is a defective virus, and cannot produce infectious progeny in the absence of RAV or of some other avian leukosis virus (Hanafusa et al., 1963). Although not entirely identical the situation with pestiviruses could be parallel with the CP component being defective. This would mean that the CP virus could not be grown in the absence of NCP virus, although procedures aimed at purifying CP virus could be expected to change the proportions of the two populations.

One other possible explanation for the distinction between CP and NCP pestiviruses is that CPE can be produced in cells infected with NCP virus in the presence of a satellite agent, either virus or nucleic acid. The case for this possibility has been argued cogently (Littlejohns and Walker, 1985), but fails to be as attractive as the case for defective and helper viruses within a pestivirus population.

Clearly the reason for the existence of NCP and CP pestiviruses, and their distribution within any virus stock is of vital importance to an understanding of the pathogenesis of MD and the equivalent disease in sheep, and

this is an important area for future work. Inconsistency among reports on the structure of pestiviruses implies that they are difficult viruses to study, but more information at the molecular level is urgently required to help to distinguish isolates with different biological properties. Western blotting procedures, as reported here, still need further development, although they may have helped to identify two important immunogenic determinants. Radioimmunoprecipitation tests with both conventional and monoclonal antibodies will provide more information as the reagents are developed.

As well as differences in the pathogenicity of virus preparations from separate sources antigenic relationships between isolates are also important. The work reported in this thesis has consistently shown that BVD viruses, particularly BVDV-NADL, are antigenically distinguishable from BDV-M in neutralisation tests using early immune sera. This, together with the results of the protection test in ewes, has re-emphasised the likely need to include more than one antigenic type in any pestivirus vaccine especially if this is to be considered for use in both cattle and sheep.

The need for such a vaccine is now well recognised (Nagele, 1984; Roeder, 1984; Barber et al., 1985). The aims of this thesis were to explore pathogenic and antigenic relationships between pestiviruses to provide information to assist with the formulation of disease control strategies. Work described here plus that reported recently elsewhere has shown that persistently infected animals hold

the key to the spread of virus within animal populations. Such animals may succumb to the effects of the virus with which they are persistently infected and may die following superinfection with some other pestiviruses, but as long as they survive they continue to be a source of infection. The major thrust of any control programme must, therefore, be the recognition of such animals and their separation from pregnant stock. An effective inactivated vaccine must be sought for use on animals before they reach breeding age.

Finally, the mechanisms involved in the maintenance of persistent pestivirus infections are an area of obvious fascination. Work reported in this thesis has demonstrated the similarities between pestivirus infections of cattle and sheep. Detailed virological and immunological assessment of such animals should contribute further to an understanding of persistent viral infections.



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## THE PATHOLOGY OF A SPONTANEOUS AND EXPERIMENTAL MUCOSAL DISEASE-LIKE SYNDROME IN SHEEP RECOVERED FROM CLINICAL BORDER DISEASE

By

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### INTRODUCTION

Border disease (BD) of lambs is caused by prenatal infection with a placenta-crossing pestivirus closely related to bovine virus diarrhoea-mucosal disease virus (BVD-MD). Three distinct forms of neuropathological change have been described, together with the conditions under which they may occur. These are, congenital hypomyelination (Markson, Terlecki, Shand, Sellers and Woods, 1959; Barlow and Gardiner, 1969), nodular periarteritis (Zakarian, Barlow and Rennie, 1975; Gardiner, Zakarian and Barlow, 1980) and necrosis and inflammation of the germinal layers of the CNS resulting in gross malformations such as hydranencephaly and cerebellar dysplasia (Clarke and Osburn, 1978; Barlow, 1980). These forms of neuropathology may be associated with abnormalities of conformation (Terlecki, Hebert and Done, 1973).

Border disease-affected lambs, commonly described as "hairy-shakers", persistently excrete virus but only rarely produce specific serum neutralizing antibody. The clinical signs resolve with time, but infected lambs are difficult to rear and the effect of weaning is drastic; many which survive to this event die when separated from their mothers. The cause of death is uncertain (Hughes, Kershaw and Shaw, 1959) but pneumonia and gastrointestinal parasitism have been implicated.

In the present report attention is drawn to characteristic pathological changes in the CNS and viscera of sheep apparently recovered from naturally-occurring and from experimental BD. Several developed intractable diarrhoea or respiratory distress with nasal discharge and died or were killed in extremis. The clinical signs and pathological changes observed were similar to those seen in calves with mucosal disease. The syndrome has been reproduced experimentally and has led to an hypothesis by means of which the pathogenic mechanisms involved can be partially explained (Gardiner, Nettleton and Barlow, 1983).

### MATERIALS AND METHODS

As summarized in Table 1, 26 animals of various breeds and crosses were involved. Although the groupings are not the same as in the accompanying paper (Gardiner *et al.*, 1983) the individual animal numbers correspond. Group I consisted of 5 animals, 3 of which were recovered "hairy shakers" (RHS) from experimentally produced BD

and 2 were from a field outbreak of the disease though they were not known to have themselves shown overt clinical signs. All 5, however, were fatally affected by a spontaneous clinical syndrome characterized by depression, persistent diarrhoea and/or nasal discharge. Groups II to IV were made up of experimentally produced RHS sheep and in each group there was a similar range of age and breed type. Group II was injected with the cytopathic virus grown from the Moredun IIB pool (Vantsis, Barlow, Fraser, Rennie and Mould, 1976), constituting a homologous challenge (since all the laboratory produced RHS sheep were the result of maternal inoculation of IIB pool). Group III was injected with the NADL strain of bovine virus diarrhoea (BVD) virus, constituting a heterologous challenge, whilst Group IV was left uninoculated. Group V consisted of the progeny of BD immune ewes which had been challenged on the 54th day of pregnancy with IIB pool. The lambs were born and raised in pen-contact with the RHS sheep but had never shown any clinical illness. Group VI were 2 normal sheep initially free of BD neutralizing antibody and were used to test the immunogenicity of the BD virus inoculum.

#### *Preparation of Inocula, Dose and Route of Administration*

Clarified BD virus suspensions containing  $10^5$  to  $10^6$  TCID<sub>50</sub> per ml were prepared from the 28th passage virus as previously described (Vantsis, Rennie, Gardiner, Wells, Barlow and Martin, 1980). BVD virus (NADL strain) of unknown passage number was grown in secondary bovine embryonic kidney cells by a similar method to the above and a clarified virus suspension containing  $10^{5.5}$  TCID<sub>50</sub> per ml was used. The suspensions were injected in 2 ml amounts subcutaneously over the shoulder.

#### *Pathology*

Animals either died or were killed by exsanguination under deep sodium pentobarbitone anaesthesia. At necropsy, the entire CNS and representative samples of other organs and tissues were fixed by immersion in Baker's calcium formol; blocks were taken, processed to paraffin, sectioned and examined by light microscopy. Stains used included HE, Luxol Fast Blue, Alcian Blue-PAS, methyl green-pyronin and Feulgen.

### RESULTS

Eight of the sheep (Table 1) developed a persistent debilitating diarrhoea, all of Group I spontaneously and 3 of Group II (nos 6, 7 and 9) 2 to 3 weeks after injection of homologous virus. The diarrhoea was watery and foetid, but examination of faeces revealed neither gastro-intestinal parasites nor pathogenic bacteria. Another 4 Group II sheep (nos 8, 10, 11 and 13) became severely depressed and one of them (no. 8) developed respiratory distress and a nasal discharge; sheep no. 13, however, subsequently recovered. The remaining Group II sheep (no. 12) and all of Groups III to VI showed no clinical malaise.

All sheep, except no. 13, died or were killed within 1 month of injection for pathological examination and virus isolation. Necropsy of sheep 13 was delayed for 10 months during which time it remained clinically normal.

At necropsy, animals in Groups V and VI were in good condition and no macroscopic abnormalities were found. In contrast, RHS sheep showed varying degrees of ill-thrift and several had enlarged spleens and lymph nodes. All the diarrhoeic sheep had lesions involving caecum and colon and extending in one

TABLE 1  
CLINICAL DISEASE AND PATHOLOGICAL FINDINGS IN SHEEP OF DIFFERING BD STATUS

Group	Status/treatment	Animal	Sex, breed	Age (months)	Clinical disease	Lesions						
						CNS	Gut	Liver	Kidney	Heart	Thyroid	Lungs
I	"Recovered hairy shakers" (RHS) Spontaneous illness	1	♂DH	20	+	—	+	—	—	—	NE	—
		2	♂DH	21	+	+	—	—	—	—	NE	+
		3	♂DH × Ch	18	+	+	NE	+	+	+	+	+
		4*	♀GF × S	2	+	NE	NE	NE	NE	NE	NE	NE
		5*	♂GF × S	3	+	NE	NE	NE	NE	NE	NE	NE
II	RHS Challenged with homologous virus (Moredun cytopathic strain of BD virus)	6	♂DH × Ch	9	+	+	—	+	+	+	NE	+
		7	♂S × Ch	10	+	+	—	+	+	+	NE	+
		8	♀BF	18	+	+	+	+	+	+	+	+
		9	♀DH	21	+	+	—	+	+	+	+	+
		10	♂DH × Ch	18	+	+	+	+	+	+	+	+
		11	♀DH	30	+	+	—	+	+	+	NE	+
		12	♂S × Ch	8	—	+	—	+	+	—	—	—
		13	♀S × Ch	18	+	+	—	—	+	—	—	—
		14	♀S × Ch	18	—	+	—	—	+	+	—	—
		15	♂DH	30	—	—	—	—	—	—	—	—
III	RHS Challenged heterologous virus (NADL BVD)	16	♂S × Ch	8	—	—	—	—	—	—	—	—
		17	♂DH × Ch	18	—	—	—	—	+	+	—	—
IV	RHS unchallenged	18	♀BF	30	—	—	—	—	+	+	—	—
		19	♀S × Ch	8	—	—	—	—	—	+	+	—
V	Normal animals from immune dams reared in contact	20	♀DH × Ch	18	—	—	—	—	—	—	NE	—
		21	♂S × Ch	18	—	—	—	—	—	—	NE	—
		22	♀S × Ch	18	—	—	—	—	—	—	NE	—
		23	♂S × Ch	18	—	—	—	—	—	—	NE	—
		24	♀S × Ch	18	—	—	—	—	—	—	NE	—
VI	Normal susceptible animals injected with live Moredun cytopathic BD virus	25	♂Ch	18	—	—	—	—	+	+	—	—
		26	♂Ch	18	—	—	—	—	—	—	—	—

DH, Dorset Horn; Ch, Cheviot; BF, Blackface; S, Suffolk; GF, Greyface.

+, Lesions present; —, lesions absent; NE, not examined.

\* From a field outbreak of BD but not known whether these individuals had themselves been hairy shakers.

or two cases for up to 10 cm into the terminal ileum. These lesions varied in intensity from slight thickening of the bowel wall to gross swelling of the muscle coats, subserous and submucous oedema, diffuse and polypoid hyperplasia of the mucous membrane, mucosal hyperaemia, ulceration and haemorrhage. Sheep nos 2, 10 and 11 had grey hepatization in the apical and cardiac lobes of the lungs.

The distribution of microscopic lesions is given in Table 1, except for sheep nos 4 and 5 which were field cases from a BD outbreak from which only brain tissue was submitted. The lesions in those RHS sheep with clinical disease were not only more widespread (Table 1) but also more severe than those in any of the other groups.

In all the affected organs (Table 1) the lesions were essentially lymphoid and proliferative. In the CNS, lymphoid infiltrations containing a variable proportion of plasma cells and macrophages were prominent in the choroid plexuses (Fig. 1). The choroidal epithelial cells were often rounded and vacuolated and some had sloughed to form cellular aggregates with lymphocytes elsewhere within the ventricular system. The periventricular brain substance showed focal and diffuse glial proliferations and perivenous lymphoid cuffings (Fig. 2).

The intestinal lesions were most marked in diarrhoeic sheep. The epithelium was hypertrophied and grossly elongated glands not infrequently breached the muscularis mucosae (Fig. 3). In sheep nos 1, 2 and 3 the epithelium of some of these ectopic glands appeared metaplastic and degenerate glandular elements were observed in submucous lymphatics (Fig. 4). The lamina propria was diffusely infiltrated with lymphoid cells and some eosinophils, causing distention of villi. Some glands were plugged with cellular detritus (Fig. 5). Focal mucosal sloughing with haemorrhage and ulceration was also encountered. The submucosa and subserosa were usually oedematous and infiltrated with lymphocytes. Muscle coats were often hypertrophied and oedematous and Meissner's plexus was unusually prominent.

Slight mucosal hypertrophy and focal submucosal and subserosal lymphoid infiltrations were present in 3 sheep (Nos 14 and 15 from Group III and No. 19 from Group IV) in which diarrhoea had not been observed.

Lesions of the liver were less common and consisted of prominent lymphoid infiltrations of the portal tracts.

The kidney lesions consisted of dense focal accumulations of lymphoid cells in the subcapsular cortex, around the vessels of the cortico-medullary junction and in the subepithelial connective tissue of the calyces (Figs 6 and 7). In the heart the lesions consisted of small foci of non-suppurative myocarditis with lymphocyte infiltration and degeneration of muscle fibres (Fig. 8).

Thyroid lesions were infrequent but severe. They consisted of heavy infiltrations of lymphoid cells associated with degeneration and obliteration of acini (Fig. 9).

In the lung the most frequently observed changes were peribronchial lymphoid infiltrations and hyperplasia of smooth muscle and bronchial and bronchiolar epithelium (Fig. 10). In sheep nos 2, 10 and 11 there were, in addition, areas of pneumonic consolidation due to macrophage infiltration or collapse of alveoli. The presence of pneumonia did not correlate with clinically evident respiratory distress, viz. sheep no. 8.

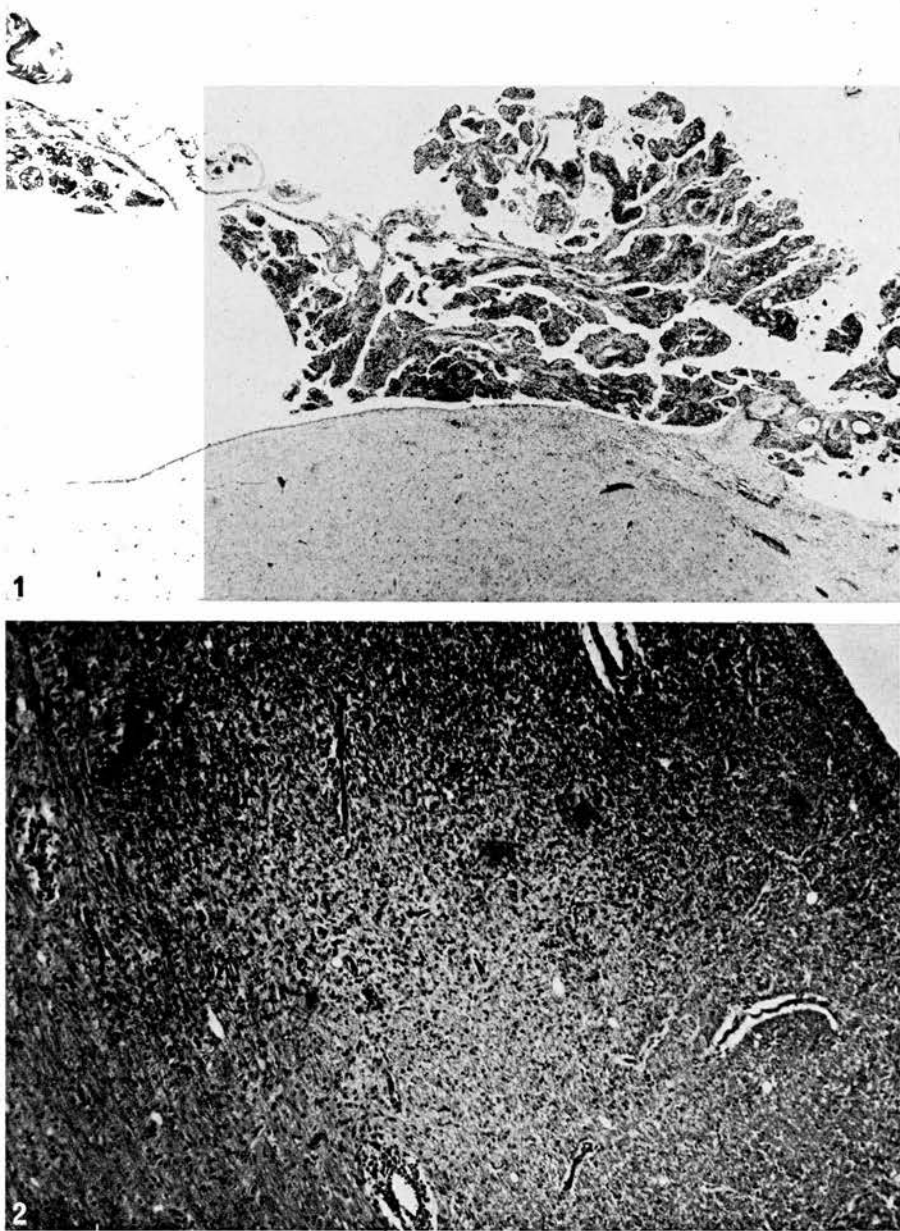
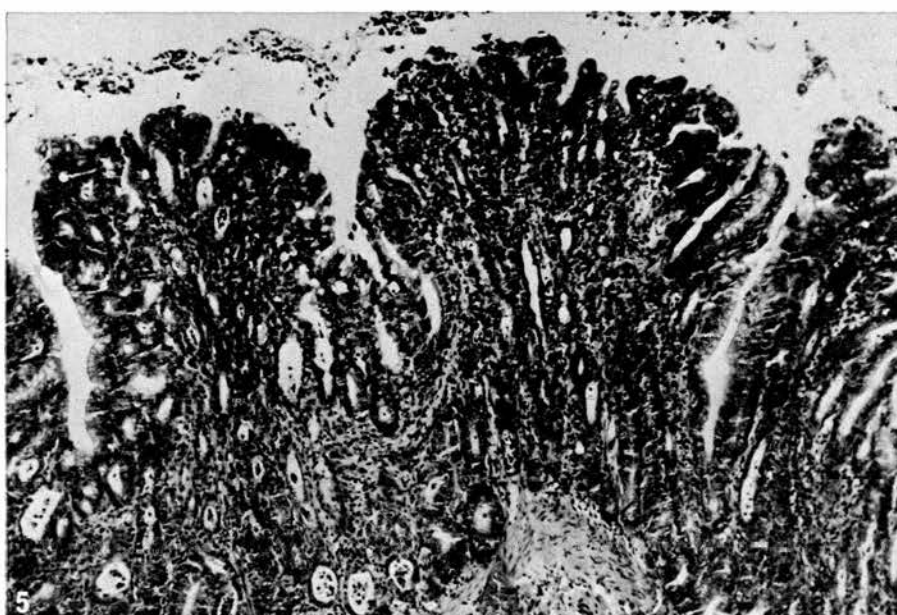
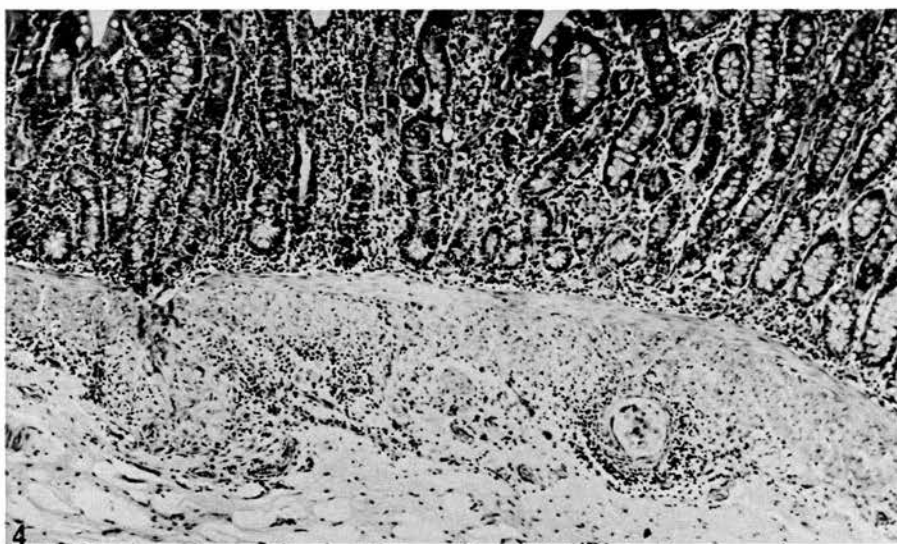
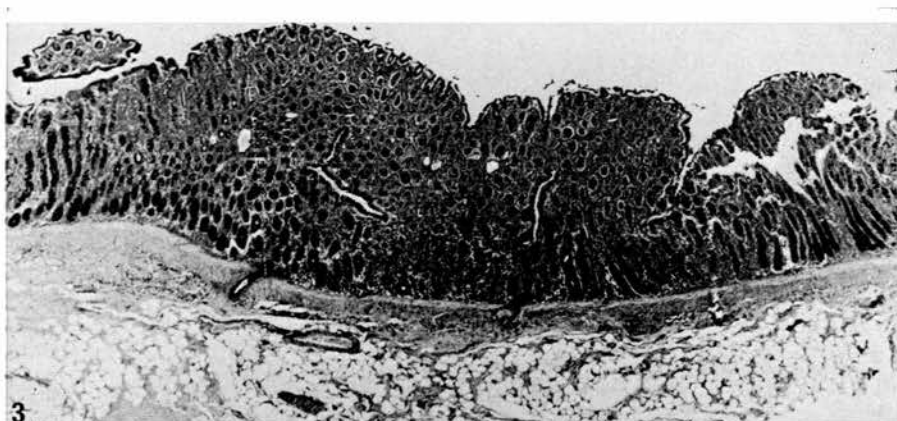


Fig. 1. Choroid plexus of 4th ventricle showing heavy infiltration by lymphoid cells. There is perivascular cuffing and diffuse proliferation of glial cells in the adjacent medulla. HE  $\times 19$ .

Fig. 2. Periventricular encephalitis with perivenous lymphoid cuffs and diffuse glial proliferation. HE  $\times 75$ .





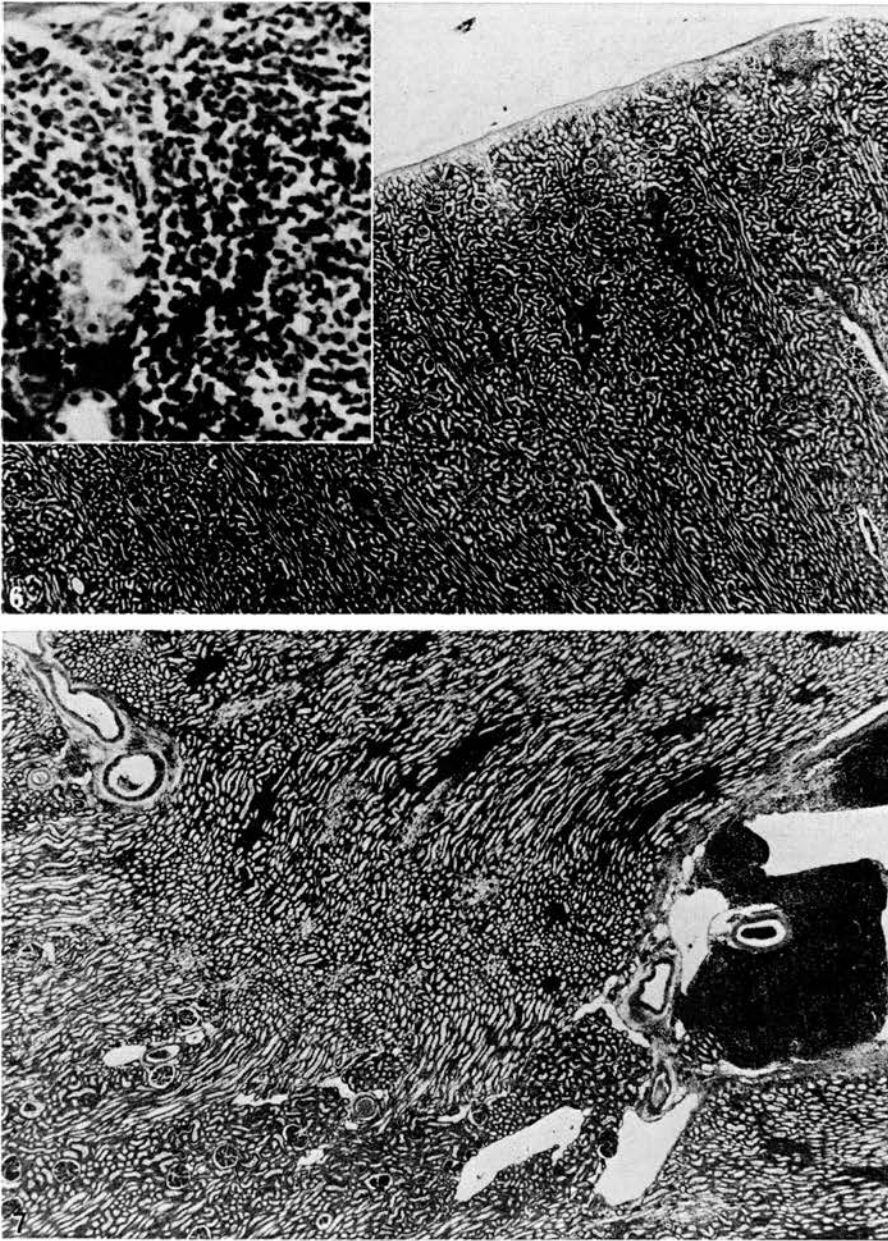


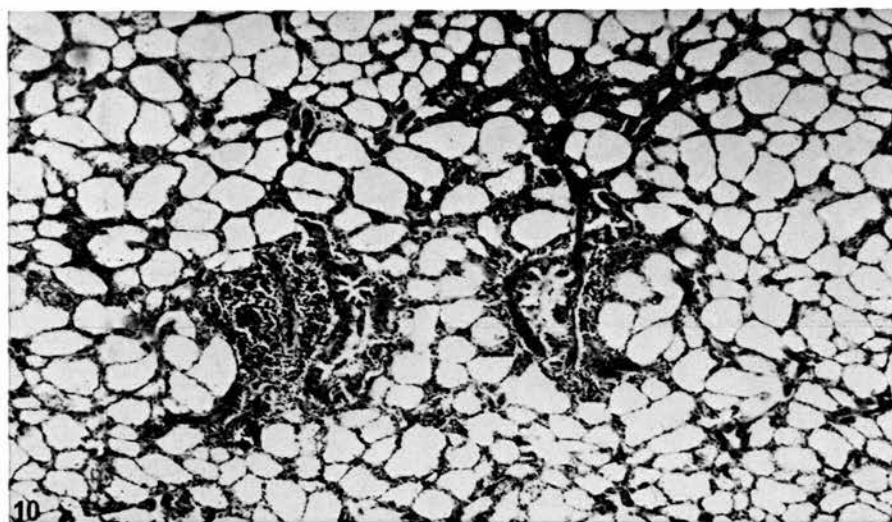
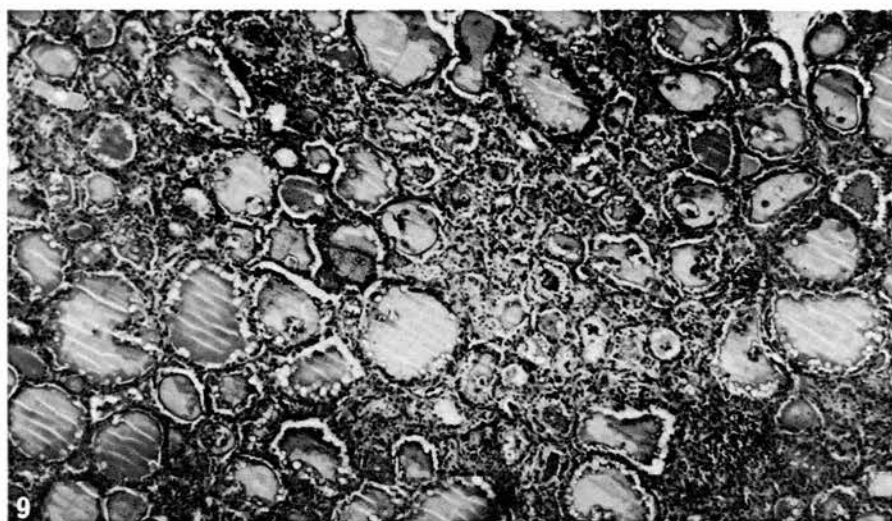
Fig. 6. Kidney showing dense focal lymphoid infiltrations in the cortex. A small artery is evident at the centre of some of the lymphoid foci. HE  $\times 19$ . Inset,  $\times 180$ .

Fig. 7. Kidney showing massive periarterial infiltrations of lymphocytes in the connective tissue of the calyces. HE  $\times 19$ .

Fig. 3. Caecum showing hypertrophy of the mucosa with penetration of the muscularis mucosa by glandular elements and severe submucous oedema. Alcian Blue-PAS  $\times 19$ .

Fig. 4. Hyperplastic typhlo-colitis. The mucosa and the oedematous submucosa are infiltrated by lymphoid cells. Degenerate glandular elements are present in a submucous lymphatic which has a lymphoid cuff (lower right centre). HE  $\times 75$ .

Fig. 5. Typhlo-colitis. The mucosa is hyperplastic with a diphtheritic membrane. Many mucosal glands are plugged with epithelial cell detritus and inflammatory cells. HE  $\times 75$ .



The white pulp of the spleen and lymph nodes was hyperplastic in animals with clinical disease but focal necrosis of lymphoid nodules was not observed.

The virological and serological results are presented in Gardiner *et al.* (1983). Suffice it to say here that virus was detected terminally in all RHS animals from which isolation was attempted but only sheep no. 13 developed a significant titre of BD neutralizing antibody. In contrast, control sheep (Groups V and VI) with one exception (no. 22) had terminal neutralizing antibody titres  $\geq 1$  in 160.

#### DISCUSSION

As can be seen from Table 1, RHS sheep in this study had lymphoid proliferations in several organs. These proliferations were most severe and widespread in those sheep which developed enteric or respiratory disease either spontaneously or following injection of homologous virus. Minor lesions of the same type were irregularly encountered in uninoculated RHS sheep, those receiving heterologous (BVD) virus and one of 2 normal susceptible adult sheep killed 4 weeks after injection with the cytopathic Moredun strain BD virus. Such lesions were not found in any of 5 normal sheep from immune mothers which had been born and raised in pen contact with the "hairy-shaker" animals. These findings strongly suggest that the lymphoid proliferations were BD-virus related, though the possibility that the RHS sheep were in some way uniquely susceptible to some other unknown infection cannot be completely excluded.

The original purpose of the injections was to determine whether apparently tolerant animals might be induced to mount an immune response and the lympho-proliferations which occurred could be interpreted in this way. However, only one RHS sheep (no. 13) clearly seroconverted, though both normal control animals did so when given the same inoculum.

Disseminated lymphoid infiltrations of the type seen here occur in some neoplastic disorders and in conditions such as malignant catarrhal fever (MCF). The heavy lymphoid infiltrations observed in kidneys and thyroids of some animals were similar to those found in MCF, though the focal necrosis of lymph nodes and the fibrinoid necrosis of the media of arteries, which are features of the latter, appeared to be absent.

Non-suppurative periventricular encephalitis in sheep with heavy lymphoid infiltrations of the choroid plexuses is a characteristic feature of infection with maedi/visna virus (Palsson, 1976) and it seemed prudent to have the sera from these animals tested for antibody by the agar gel immunodiffusion test. None was positive. CNS lesions of this type have been encountered in neuropathological diagnostic material (unpublished observations) and their presence here in sheep from a farm experiencing BD, as well as in laboratory-produced RHS sheep, suggests that periventricular encephalitis may be an indication of previously undiagnosed BD infection.

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Fig. 8. Heart muscle with a focus of non-suppurative myocarditis; lymphoid infiltration and necrosis of muscle fibres. HE  $\times 75$ .

Fig. 9. Thyroid with interstitial lymphoid infiltration and degeneration and obliteration of acini. HE  $\times 75$ .

Fig. 10. Lung showing mild interstitial pneumonia and prominent peribronchial and perivascular lymphoid infiltrations. HE  $\times 75$ .



The pulmonary changes observed—peribronchial lymphoid infiltrations, and hyperplasia of bronchial epithelium and smooth muscle—are also features of maedi/visna and of ovine atypical or proliferative exudative pneumonia (Stamp and Nisbet, 1963; Gilmour, Jones and Rae, 1979). The latter is a common disorder of sheep in these age groups and the possibility that the pulmonary changes represent intercurrent infection cannot be excluded.

The enteric lesions are more specific. Though the hyperplastic epithelium and the glandular penetration of the submucosa with extension into lymphatics are tumour-like properties, reminiscent of the porcine adenomatosis complex (Rowland, Lawson and Maxwell, 1973), this typhlo-colitis can be distinguished histologically from most other forms of ovine enteritis. The ulcerative, haemorrhagic changes in a hyperplastic, heavily infiltrated mucosa with crypt abscesses closely resemble the changes seen in calves with mucosal disease. Whilst the possibility exists that the lesions seen here are due to some unsuspected intercurrent infection, it seems probable that the lympho-proliferative disorder described represents a further, newly recognized component of the pathology of BD infection. As such it links the disease even more closely to BVD.

In conclusion, we suggest that the lympho-proliferative disease in these RHS sheep was triggered by a disturbance in the virus-host relationship in apparently tolerant, persistently infected animals. Though the mechanisms are unclear it is possible that the lesion represented a florid, immune-type cellular response which was usually ineffective, though one sheep did seroconvert and recover.

#### SUMMARY

The pathological changes which accompanied a severe clinical syndrome characterized by intractable diarrhoea and/or respiratory distress in sheep recovered from clinical Border disease consisted of inflammatory lympho-proliferative lesions in several organs, notably the CNS and intestinal tract. The syndrome has been reproduced experimentally by "super-infection" with homologous Border disease virus. The differential diagnosis is discussed and attention drawn to the similarity between the enteric lesions and those of bovine mucosal disease.

#### ACKNOWLEDGMENTS

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## VIROLOGY AND IMMUNOLOGY OF A SPONTANEOUS AND EXPERIMENTAL MUCOSAL DISEASE-LIKE SYNDROME IN SHEEP RECOVERED FROM CLINICAL BORDER DISEASE

By

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### INTRODUCTION

A new virus-related phenomenon has been described in young sheep clinically recovered from Border disease (BD) (Barlow, Gardiner and Nettleton, 1983). In this paper the virological and serological findings are presented and their significance discussed in relation to mucosal disease (MD) of cattle, a fatal form of bovine virus diarrhoea (BVD) virus infection.

Border disease-affected lambs ("hairy shakers") result from transplacental infection occurring during, approximately, the first half of gestation. They remain persistent excretors of virus for several years but rarely develop neutralizing antibodies (Plant, Gard and Acland, 1977; Vantsis, Linklater, Rennie and Barlow, 1979). Such animals may be immuno-tolerant to the virus.

To test the stability and specificity of this putative immune tolerance, groups of recovered hairy shaker (RHS) sheep were challenged with live homologous (BD) or heterologous (NADL strain BVD) virus. The results of homologous challenge were dramatic and paralleled spontaneous but previously unexplained disease observed in RHS sheep.

### MATERIALS AND METHODS

#### *Animals*

As summarized in Table 1, 17 RHS and 4 susceptible normal sheep were examined. Although the groupings are not the same as in the previous paper (Barlow *et al.*, 1983) individual animal numbers correspond. All the RHS had been produced by inoculating their dams at 54 days gestation with IIB BD brain pool (Barlow, 1972). Group 1 comprised 3 RHS sheep in which a clinical syndrome characterized by depression, with persistent diarrhoea or nasal discharge, had occurred spontaneously (Barlow *et al.*, 1983). Groups 2 and 3 were inoculated respectively with (a) the cytopathic Moredun strain BD tissue culture virus grown from the IIB pool (Vantsis, Barlow, Fraser, Rennie and Mould, 1976) and (b) the NADL strain of BVD virus, which is also cytopathic, representing homologous and heterologous challenges, respectively. Group IV consisted of untreated RHS sheep while Groups V and VI were normal sheep initially free of BD and BVD neutralizing antibodies used to test the immunogenicity of the 2 virus inocula.

#### *Preparation of Inocula, Dose and Route of Administration*

BD virus for animal inoculation was grown on primary foetal lamb kidney (FLK) 0021-9975/83/030463+07 \$03.00/0

TABLE I  
CLINICAL DISEASE, VIROLOGICAL AND IMMUNOLOGICAL FINDINGS IN SHEEP OF DIFFERING BD STATUS

Group	Status and treatment	Animal no.	Age in months	Clinical disease	Lympho-proliferation	Antibody* vs.		Virus isolations		
						BD	BVD	No. of tissues examined	N	C
I	Recovered hairy shakers (RHS) spontaneous illness	1	20	+	+	—	—	5	1	3
		2	21	+	+	7	—	7	4	3
		3	18	+	+	—	—	11	0	8
II	RHS challenged with homologous virus (Moredun cytopathic strain of BD virus, Vantsis <i>et al.</i> (1976))	6	9	+	+	7	—	23	5	14
		7	10	+	+	—	—	4	4	4
		8	18	+	+	12	—	9	0	9
		9	21	+	+	12	—	8	0	7
		10	18	+	+	—	—	10	0	10
		11	30	+	+	—	—	10	1	7
		12	8	—	—	—	—	7	1	5
		13	18	+	±	16	—	8	3	4
						—	—	ND	ND	ND
								56	5	46
III	RHS challenged with heterologous virus (NADL BVD)	14	18	—	±	—	5	7	4	0
		15	30	—	±	—	15	7	2	3
		16	8	—	—	—	25	7	7	0
								21	13	3
IV	RHS unchallenged	17	18	—	±	—	—	7	5	0
		18	30	—	—	—	—	7	3	0
		19	8	—	±	—	—	7	4	0
V	Susceptible animals challenged with live Moredun cytopathic BD virus	25	18	—	—	335	7	21	12	0
		26	18	—	—	335	5	6	0	0
								6	0	0
VI	Susceptible animals challenged with live NADL BVD virus	27	18	—	ND	9	35	12	0	0
		28	18	—	ND	—	24	ND	ND	ND
								ND	ND	ND

\*Antibody titres of animals 1 to 12 and 14 to 19 at time of death or necropsy and of animals 13 and 25 to 28, 3 weeks post inoculation. —Indicates titre < 4;  
= non-cytopathic virus isolations; C=cytopathic virus isolations.

cells from the 28th passage virus as previously described (Vantsis, Rennie, Gardiner, Wells, Barlow and Martin, 1980). Clarified virus suspensions containing  $10^5$  to  $10^6$  TCID<sub>50</sub> per ml were used as inocula. BVD virus (NADL strain) of unknown passage number was grown in secondary bovine embryonic kidney cells by a similar method to the above and a clarified virus suspension containing  $10^{5.5}$  TCID<sub>50</sub> per ml was used for experimental infection.

Animals received a 2 ml inoculation of live virus subcutaneously over the left shoulder.

#### *Virus Isolation*

Samples of between 4 and 11 tissues from each animal were collected aseptically into virus transport medium and subsequently inoculated into duplicate tubes of secondary FLK cells previously screened and shown to be free of contaminating pestiviruses (Nettleton, Herring and Corrigan, 1980). The cells were incubated at 37°C and examined regularly for virus cytopathic effect (CPE). After 7 days cultures were frozen and thawed and passaged into further tubes of FLK cells. All specimens received a minimum of 3 such weekly passages and viruses which had caused complete destruction of the cell sheet after this time were classified as cytopathic. Non-cytopathic pestiviruses were detected by an indirect immunofluorescent test with a convalescent sheep serum with a high titre against BD virus (Gardiner, 1980) and the identity of all the isolates was confirmed by this method.

#### *Serology*

Heat inactivated serum samples were tested for the presence of antibodies in a micro-neutralization test against both BD and BVD viruses with approximately 100 TCID<sub>50</sub> of virus per well. Serum dilutions and virus were incubated for 1 h at 37°C before the addition of cells from a semi-continuous fibroblastic cell line grown from foetal lamb brain. Cultures were examined for the presence of CPE after 5 days and the result expressed as the reciprocal of the serum dilution corresponding to the 50 per cent end-point of neutralization as calculated by the Spearman-Kärber method (Lennette and Schmidt, 1969). The results quoted are the means of at least 3 separate tests against each of the viruses.

### RESULTS

Serological and virological results are summarized in Table 1 together with the occurrence of clinical disease. Detailed pathological findings are to be found in the previous paper (Barlow *et al.*, 1983).

#### *Clinical*

Six sheep developed an intractable debilitating scour, nos 1, 2 and 3 spontaneously and 6, 7 and 9, 2 to 3 weeks after the injection of homologous live virus. Examination of the faeces revealed no evidence of gastro-intestinal parasites or pathogenic bacteria. Four of the sheep injected with live homologous virus (nos 8, 10, 11 and 13) became severely depressed, no. 8 developing mucous nasal discharge and mild respiratory distress. No sheep in any of the other injected groups displayed any clinical malaise. All the sheep except no. 13 died or were killed within a month of injection. As no. 13 showed signs of recovery, necropsy was delayed for 10 months during which period it remained clinically normal.

### *Serology*

In normal sheep, both challenge viruses stimulated the production of serum neutralizing (SN) antibodies with a degree of cross-reactivity by 3 weeks after inoculation but BD virus appeared to be more immunogenic. However, the antibody produced by RHS sheep after challenge did not cross-react; groups 1 and 2 produced only anti-BD antibodies and group 3 only anti-BVD antibodies. Sheep no. 13 was negative at 3 weeks but subsequently seroconverted specifically, having anti-BD titres of 47, 110 and 73 at 2, 4 and 8 months after inoculation, respectively, whilst anti-BVD antibodies remained undetectable over this period.

### *Virology*

No virus was detected in any of the 12 tissues examined from the 2 normal sheep 3 weeks after inoculation with live BD virus but virus was isolated from all the RHS sheep. In those groups in which clinical disease occurred (Table 1) virus was predominantly cytopathic and was isolated from a larger range of tissues.

### DISCUSSION

Clinical disease associated with widespread and severe lympho-proliferative changes occurred either spontaneously or after "superinfection" with the homologous BD virus but not with the heterologous BVD virus. Although these changes suggest an immunological response, they were associated with little or no neutralizing antibody and virus was not eliminated, and it is possible that the immune system was specifically compromised by exposure to the same virus during foetal life. Most affected lambs and RHS persistently excrete virus and the distribution of BD viral antigen is comparatively stable over long periods (Gardiner, 1980). Thus a type of dynamic equilibrium exists between virus and host.

The virological results in groups 3 and 4 confirm our experience that BD virus isolated from RHS lambs and sheep is almost always non-cytopathic in tissue culture, even when the original infecting virus was cytopathic. However, in the groups of animals with clinical signs of disease, isolates of virus were predominantly cytopathic. In the experimental cases re-isolation of the cytopathic super-infecting virus may have occurred but a more generally valid explanation would be that changes, reflected as regained cytopathogenicity in culture, occurred in the persisting virus. The state of equilibrium in the apparently healthy RHS lamb may therefore reflect some form of virus attenuation within host cells, disease arising from an upset of this virus/host equilibrium, with reassertion of viral pathogenicity.

The disease syndrome observed in these sheep bears a strong resemblance to aspects of BVD infection in cattle, notably the fatal form known as mucosal disease (Malmquist 1968; Steck, Lazary, Fey, Wandeler, Huggler, Oppliger, Baumberger, Kaderli and Martig, 1980). Antigenic cross-reactivity occurs between BD, BVD and swine fever/hog cholera viruses (Acland, Gard and Plant,

1972; Hamilton and Timoney, 1972; Plant, Littlejohns, Gardiner, Vantsis and Huck, 1973) and similar teratogenic consequences of infecting sheep with BVD virus and cattle with BD virus have been recorded (Barlow, Rennie, Gardiner and Vantsis, 1980; Gibbons, Winkler, Shaw, Terlecki, Richardson and Done, 1974). In BVD, changes in cytopathogenicity have also been reported (Malmquist, 1968; Done, Terlecki, Richardson, Harkness, Sands, Patterson, Sweasey, Shaw, Winkler and Duffell, 1980). Thus it is clear that BD and BVD have much in common, differences being more of degree than of a fundamental nature.

Persistence of virus and lack of antibodies following foetal infection are indicators of immune tolerance or suppression. Malmquist (1968) and Steck *et al.* (1980) have associated MD or chronic BVD with prenatal infection and possible immune tolerance. Johnson and Muscoplat (1973) and Muscoplat, Johnson and Teuscher (1973) have presented evidence for immunosuppression and upsets in lymphocyte function in young cattle brought in from the field suffering from chronic BVD infection. Immunosuppression associated with acute BVD infections and suppressive effects of the virus on lymphocyte cultures have also been described (Muscoplat, Johnson and Stevens, 1973; Pospisil, Machatkova, Mensik, Rodak and Muller, 1975; Roth, Kaeberle and Griffith, 1981; Reggiardo and Kaeberle, 1981).

A hypothetical concept of immune tolerance in relation to the present work is illustrated in Fig. 1. If an animal were completely tolerant to virus SA, reactivity to all the shared antigens enclosed by the broken line would be absent. Subsequent immune response to viruses SB, SC and SD would be directed against B or C or D antigens only, i.e. tolerance confers specificity on a partially cross-reacting system.

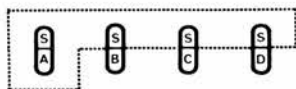


Fig. 1. A hypothetical series of viruses with shared (S) and unshared (A, B, C, D) antigens. Tolerance to virus SA confers non-reactivity to the shared antigenic components of the other viruses enclosed within the broken line.

The experiments reported here support this theory. Lambs were specifically susceptible to superinfection with the strain of virus they were excreting and to which, up to the time of challenge, they had no SN antibodies. Following challenge there was an apparent breakdown in tolerance in 4 out of 8 animals but antibodies cross-reacting with BVD virus were not detected. The antibody produced by RHS sheep challenged with BVD virus did not cross-react with BD virus. Had the animals lived longer, cross-reacting antibodies might have appeared, but in the one animal (no. 13) that survived for 10 months this did not happen.

These results are relevant to poorly explained aspects of mucosal disease viz. the occurrence of spontaneous illness and death in persistent virus excretors in the presence or absence of SN antibody. A recent report on the vaccination of 209 seronegative cattle with a live Oregon strain BVD virus vaccine (Steck *et*

*al.*, 1980) is typical. Four animals persistently excreted a field strain of BVD virus to which they produced no SN antibody either before or after vaccination. Although they responded specifically and well to the vaccine, they subsequently died of MD. Two further animals did not respond to the vaccine and died 8 and 16 days later, MD being diagnosed in one. Such results have serious implications for vaccination. Non-reactors, or those producing non-cross-reacting antibody, may be immunotolerant persistent excretors of virus and may die from mucosal disease subsequently.

In view of the foregoing we conclude that sheep and cattle persistently excreting BD or BVD viruses are likely to be specifically immunotolerant to a particular strain of virus as a result of transplacental infection with that strain in early pregnancy. In such animals the virus appears to exist in equilibrium with the host in some attenuated form. Pathogenicity may be reasserted following specific homologous re-infection or following some unknown event, the consequence being mucosal disease in cattle and a similar syndrome in sheep.

#### SUMMARY

A disease similar to mucosal disease (MD) of cattle has occurred spontaneously in sheep clinically recovered from Border disease (BD). Evidence is presented to suggest that, in such animals, a specific and dynamic equilibrium exists between an attenuated form of the virus and the immunotolerant host. Upset of this equilibrium either by injection of BD virus of the same strain or by some unknown spontaneous event appears to lead to the reassertion of viral pathogenicity with fatal consequences.

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